# Oxidative Stress in Cucumber (*Cucumis sativus* L.) Seedlings Treated with Acifluorfen

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Illumination of acifluorfen-sprayed and 14 h-dark-incubated cucumber seedlings with cool-white fluorescent light caused overaccumulation of protoporphyrin IX and severe oxidative stress which resulted in seedling death within 72 h. The ratio of variable fluorescence ($F_v$)/maximum fluorescence ($F_m$), a measure of the functional status of PSII, declined by 30%, 58% and 85% after 24 h, 48 h and 72 h respectively. When 24 h-light-treated plants were transferred to dark for 24 h the $F_v/F_m$ ratio was restored to near control levels suggesting a dark recovery from photodynamic damage. The intracellular distribution of protoporphyrin IX that accumulated in the cotyledons of acifluorfen-treated and 1 h-light-exposed plants was 22% within and 78% outside chloroplasts suggesting migration of protoporphyrin IX within one hour of exposure of plants to light. When thylakoid membranes, treated with exogenous protoporphyrin IX, were illuminated there was a reduction in photosystem II activity. Addition of L-histidine, scavenger of singlet oxygen, to illuminated thylakoid+ protoporphyrin IX mixture resulted in substantial protection of photosystem II activity suggesting the involvement of singlet oxygen in protoporphyrin IX-mediated photodynamic damage. In illuminated thylakoid+protoporphyrin IX mixture no indication was found for production of superoxide radicals via type I reaction. In cucumber seedlings acifluorfen-induced synthesis of protoporphyrin IX caused severe photodynamic damage mediated by singlet oxygen generated due to type II photosensitization reaction.

## Introduction

Substances that require direct interaction with light and molecular oxygen to produce active oxygen species are known as photodynamic compounds. There are two main types of porphyrin-generating compounds. One consists of 5-aminolevulinic acid (ALA) (Rebeiz *et al.,* 1984, 1988; Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992; Tripathy, 1994) and the other consists of diphenyl ethers (DPEs) and allied substances which inhibit protoporhyrinogen oxidase (Protox) (Matringe and Scalla, 1988; Witkowski and Halling, 1988).

DPEs and allied substances have multiple modes of action at different concentrations (Böger, 1984). Usually they induce the accumulation of protoporphyrin IX (Proto IX) in plants. DPEs inhibit protoporphyrinogen oxidase (Protox) which is the last enzyme of the common branch of the heme- and chlorophyll-synthetic pathways in plants (Matringe and Scalla, 1988; Duke *et al.,* 1990). The light dependent lethal effect of DPEs are a result of lipid peroxidation, which leads to destruction of polyunsaturated fatty acids in membranes (Duke and Rebeiz, 1994). Initiation of peroxidation of membrane lipids due to herbicide and light treatment depends on generation of toxic active oxygen species. Differential tolerance of various species to DPE herbicides may be due to differences in rates of herbicide absorption, Proto IX accumulation and antioxidants present in the plant cell (Matsumoto *et al.,* 1994).

In the present study the technical grade material acifluorfen-sodium (AF-Na) (Rohm and Haas) was used to investigate the mechanism of action of

*Abbreviations:* AF-Na, acifluorfen-sodium; ALA, 5-aminolevulinic acid; DCIP, 2,6-dichlorophenol indophenol; DPE, diphenyl ether; MV, methyl viologen; PD, *p*-phenylenediamine; Protox, protoporphyrinogen oxidase; proto IX, protoporphyrin IX; protogen IX, protoporphyrinogen IX; SOD, superoxide dismutase; TIRON, 1,2-dihydroxybenzene-3, 5-disulfonic acid.

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Proto IX-mediated photodynamic damage to the photosynthetic electron transport chain and to identify the toxic active oxygen species involved in photodynamic reactions.

## Materials and Methods

Cucumber (*Cucumis sativus* L. cv Poinsette) seedlings were grown on moist germination paper in Petri plates (14.5 cm diameter), under continuous cool white fluorescent light. Temperature was maintained at 25 °C. Five-day-old seedlings were used in all experiments.

The technical grade material AF-Na was a gift from Rohm and Haas Bayport Inc. USA. Technical grade material AF-Na was diluted with water+0.5% Tween-20 and each Petri plate having a population of 25 seedlings was sprayed with 10 ml of 80 μM of AF-Na. A glass sprayer (atomizer) attached to a rubber bulb was used for spraying AF-Na. Control seedlings were sprayed with distilled water having 0.5% Tween-20. After spraying plants were covered with aluminum foil and kept for 14 h in darkness at 25 °C, prior to light exposure. Plants were exposed to different intensities of cool white fluorescent light at 25 °C. Light intensity was measured using LI-COR Quantum meter Model LI-185B with a LI-190SB quantum sensor (LI-COR Inc., Lincoln, USA).

Chloroplasts were isolated from cucumber cotyledons at 4 °C, under safe green light, by hand-homogenizing the tissue in an isolation medium consisting of 0.4 M sucrose, 10 mM NaCl and 25 mM Hepes-NaOH (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (pH 7.6). After filtration of the homogenized tissue through 2 layers of Mira cloth, it was centrifuged at 3,000×*g* for 5 min to sediment the chloroplasts. Chloroplasts were suspended in isolation buffer and intact chloroplasts were obtained using a 50% (v/v) percoll gradient (Chakraborty and Tripathy, 1992). The final intact chloroplast pellet was suspended in a minimal volume of the isolation buffer. Thylakoid membranes were prepared from chloroplasts by giving an osmotic shock. This was done by diluting chloroplasts 10-fold with 20 mM Hepes-NaOH buffer (pH 7.6). Thylakoid membranes were sedimented by centrifugation at 3,000×*g* for 5 min and the pellet was suspended in isolation medium. Chlorophyll (Chl) (Porra *et al.*, 1989) and carotenoid (Wellburn and Lichtenthaler, 1984) contents were estimated in 80% acetone.

Thylakoid membrane suspensions (1 mg Chl $ml^{-1}$) in the absence (control) or presence (treated) of exogenously added Proto IX, were illuminated in a glass tube surrounded by a water jacket connected to a circulating water bath (Julabo F 10, Julabo Labortechnik GmBH, Germany). Thylakoid membranes were illuminated with white light from a tungsten light source at a photon flux rate of 250 μmol $m^{-2}$ $s^{-1}$, which was obtained by passing the light through neutral density filters.

Assays of electron transport activity of photosystems (PS)II and PSI were carried out using a glass cuvette fitted within a Clark-type oxygen electrode (YSI, Yellow Springs, USA). The reaction mixture was maintained at 25 °C by using a temperature controlled water bath and was illuminated for 20 seconds with white light from a tungsten light source at a photon flux rate of 1500 μmol $m^{-2}$ $s^{-1}$. The reaction mixture (3 ml) for *p*-phenylenediamine (PD)-supported and PSII-mediated $O_2$ evolution consisted of 50 mM Hepes/NaOH (pH 7.6), 3 mM $MgCl_2$, 10 mM NaCl, 1 mM $K_3Fe(CN)_6$ and 0.3 mM freshly prepared PD (Tripathy and Chakraborty, 1991). Thylakoids were added at concentration of 10 μg Chl $ml^{-1}$ to the assay medium. The partial electron transport chain through PSI was measured as oxygen consumption (Tripathy and Mohanty, 1980). Electron flow from PSII was blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Ascorbate/ DCIP couple was used as electron donor to PSI and MV was used as electron acceptor. Assay medium (3 ml) contained thylakoids (10 μg Chl $ml^{-1}$), 50 mM Hepes (pH 7.6), 3 mM $MgCl_2$, 10 mM NaCl, 1 mM $NH_4Cl$, 1 mM $NaN_3$, 0.5 mM MV, 20 μM DCMU, 1 mM sodium ascorbate and 100 μM DCIP.

Fluorescence transients of intact leaves or thylakoids were monitored by pulse amplitude modulated (PAM) Chl fluorometer (PAM 101, Heinz Walz, Germany) (Schreiber, 1986). For measurement of $F_o$, modulated light of 1.6 KHz with light intensity setting at 6 (0.075 μmol $m^{-2}$ $s^{-1}$), gain at 12 and damping at 11 were used. For measurement of $F_m$, modulated light of 100 KHz along with actinic light (900 μmol $m^{-2}$ $s^{-1}$) was used for 15 seconds, while keeping the other settings same as that for $F_o$ measurement. Light is communicated

from the source to the sample through a Walz fiber optic cable (type 101F). Leaves and thylakoid membranes were dark adapted for 15 min before fluorescence transients were recorded. For measuring transients of thylakoid samples, isolated thylakoids at a concentration of 15 μg Chl $ml^{-1}$ were suspended in a buffer containing 50 mM sucrose and 10 mM Hepes (pH 7.6). To measure the effect of exogenous electron donors on fluorescence transients, the thylakoids (15 μg Chl $ml^{-1}$) were suspended in a buffer containing 50 mM sucrose, 10 mM Hepes (pH 7.6) and 3.6 mM $MgCl_2$. Exogenous electron donors, freshly prepared diphenylcarbazide (DPC), $NH_2OH$ (neutralized to pH 6.8) or $MnCl_2$ at final concentrations of 0.5 mM, 10 mM and 0.3 mM, respectively were added to the thylakoid suspension.

For the estimation of tetrapyrroles, tissues were hand-homogenized in 90% cold ammoniacal acetone (acetone : 0.1 N $NH_4OH$ = 9:1 v/v) under safe green light. Fully esterified tetrapyrroles were extracted with equal volume of hexane. While the mono- and di-carboxylic tetrapyrroles remained in the hexane-extracted acetone residue (HEAR) solvent mixture fraction, the fully esterified tetrapyrroles were transferred to hexane phase. Quantitative estimation of Proto IX and Pchlide from their mixture in HEAR was carried out spectrofluorometrically (Hukmani and Tripathy, 1992; Tewari and Tripathy, 1998). Fluorescence spectra of HEAR fractions were recorded in the ratio mode using a computer-driven SLM AMINCO 8000C spectrofluorometer and corrected for photomultiplier tube sensitivity. Rhodamine B was used in the reference channel as a quantum counter. A tetraphenylbutadiene block was used to adjust the voltage in both the sample as well as reference channels, to 20,000 counts/sec. The block was excited at 348 nm and emission was monitored at 422 nm. Spectra were recorded at excitation and emission bandwidths of 4 nm. Emission spectra were recorded from 580 to 700 nm.

A spectrophotometric assay was used to determine the superoxide content (Asada, 1984). Thylakoid membranes were prepared and to remove stromal superoxide dismutase, the thylakoids were incubated for 1 h at 4 °C in 50 mM potassium phosphate (pH 7.6) having 1 mM ethylenediaminetetraacetic acid (EDTA). This washing procedure was repeated once more, and centrifuged (3000×*g* for 5 min) to pellet the thylakoid membranes. The pellet was suspended in 50 mM potassium phosphate (pH 7.8). Superoxide production was determined by monitoring cytochrome C reduction at 550 nm using an extinction coefficient of 19 $mM^{-1}$ $cm^{-1}$, and its amount calculated using absorbance coefficient of 19 $mM^{-1}$ $cm^{-1}$. The assay mixture (3 ml) contained 50 mM potassium phosphate (pH 7.6), 10 mM NaCl, 150 μM ferricytochrome C, and washed thylakoid membranes (50 μg Chl). Absorbance of this reaction mixture was taken as the initial absorbance. Subsequently, the reaction mixture was illuminated at 1500 μmol $m^{-2}$ $s^{-1}$ for 20 seconds and the increase in absorbance due to reduction of cytochrome C over the initial absorbance was used for calculating $O_2^-$ content.

## Results

### *Effect of different light intensities on pigment contents*

Light was mandatory for AF-Na-induced photodynamic damage of tissue and there was no damage in AF-Na-sprayed plants kept in dark for 72 h. AF-Na-treated plants incubated in dark for 14 h and subsequently transferred to different intensities of coolwhite fluorescent light (50, 250 and 500 μmol $m^{-2}$ $s^{-1}$) developed necrotic spots within 24 h and were severely damaged by 72 h, as a consequence of photodynamic damage. As the light intensity increased from 50 to 500 μmol $m^{-2}$ $s^{-1}$ and the light exposure period increased from 24–72 h, Chl a (Fig. 1A), Chl b (Fig. 1 B), total Chl (Fig. 1C) and carotenoid (Fig. 1D) contents decreased progressively. The pigment contents of cotyledons were corrected for the loss of moisture content. These demonstrated that the AF-Na-induced photodynamic damage was dependent on light intensity.

### *Impairment of photosynthetic electron transport due to photodynamic damage and its recovery in dark*

PSII, is very sensitive to slight perturbation of thylakoid membrane structure under stress conditions. Therefore, any damage to thylakoid membrane is likely to affect the activity of PSII. The Chl *a* fluorescence induction kinetics of leaves is an excellent endogenous (non-invasive) probe of

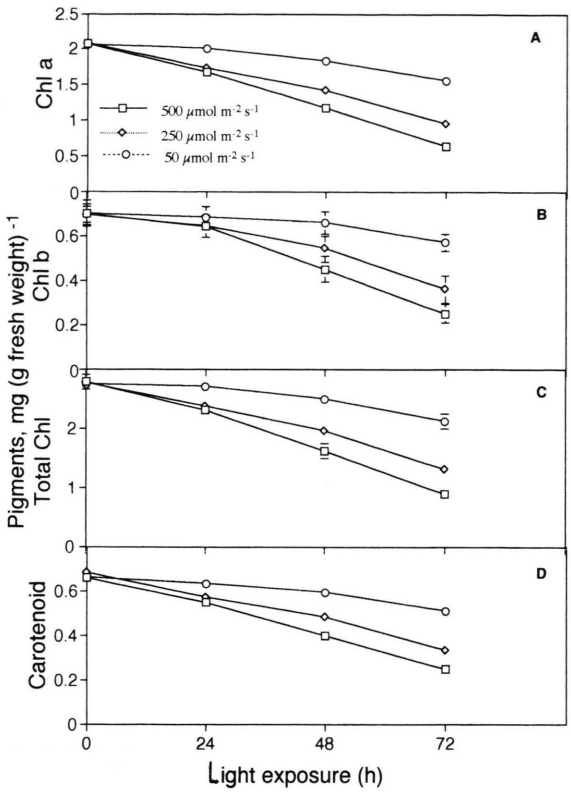


Fig. 1. Loss of Chl *a* (A), Chl *b* (B), total Chl (C) and carotenoid (D) contents in cotyledons of AF-Na-treated cucumber plants exposed to cool-white fluorescent light of different intensities (50, 250 and 500 μmol $m^{-2}$ $s^{-1}$) for 24–72 h. Pigment contents were corrected for decrease in moisture content. Each observation is the mean of 5 replicates and error bars represent SD. Missing error bars indicate that they are smaller than the the symbol.

the functional status of the photosynthetic electron transport chain and especially that of PSII. The ratio of variable fluorescence ($F_v$)/maximum fluorescence ($F_m$) is a measure of the functional status of PSII. AF-Na-treated and 14 h dark-incubated plants were transferred to cool white fluorescent light (75 μmol $m^{-2}$ $s^{-1}$) for 24–72 h and the Chl a fluorescence transient of leaves was monitored. After 24 h , 48 h and 72 h light exposure, the $F_v/F_m$ ratio of leaves declined by 30%, 58% and 85% respectively (Table I). In order to study the reversibility of photodynamic damage AF-Na-treated plants were exposed to light (75 μmol $m^{-2}$ $s^{-1}$) for 24 to 72 h and then transferred to dark for 24 h and the Chl a fluorescence transient of leaves was monitored. The $F_v/F_m$ ratio in 24h-light exposed AF-Na-treated plants declined by 0.25 (0.83–0.58). When 24 h-light-treated plants were transferred to dark for 24 h the PSII activity partially recovered and consequently the $F_v/F_m$ ratio increased from 0.58 to 0.77 i.e., its inhibition from control was 0.83–0.77=0.06 . Therefore the recovery in the $F_v/F_m$ ratio was 76% (0.25–0.06/0.25×100) (Table I). When AF-Na-treated plants were exposed to continuous light for 48 and 72 h, there was severe damage to plants but when these plants were transferred to dark for 24 h, further damage was prevented. However, there was only marginal recovery from photodynamic damage as indicated by only slight restoration of the inhibition in $F_v/F_m$ ratio. Exposure of AF-Na-treated cucumber plants to higher light intensity (500 μmol $m^{-2}$ $s^{-1}$) resulted in loss of $F_v/F_m$ ratio by 70% within 24 h and there was no recovery after these plants were returned to dark for 24 h.

### *Proto IX accumulation due to AF-Na treatment*

Untreated cucumber seedlings exposed to cool white fluorescent light (50 μmol $m^{-2}$ $s^{-1}$) does not accumulate Proto IX. However, AF-Na-treated seedlings after being exposed to light accumulated substantial amounts of Proto IX (Fig. 2). The amount of accumulated Proto IX declined after 72 h of light exposure. Proto IX does not accumulate in AF-Na-treated plants in dark (Nandihalli *et al.*, 1991). The exact mechanism of light-induced accumulation of Proto IX in AF-Na-treated plants is not understood.

### *Plastidic and extraplastidic distribution of Proto IX formed in response to AF-Na treatment*

It is proposed that in response to AF-Na and light treatment Protogen IX migrates to cytoplasm and attaches to plasma membrane where it is oxidized to Proto IX by AF-Na-insensitive protox-like enzymatic activity in plasma membrane (Jacobs *et al.*, 1991). In order to verify this hypothesis and to quantify the amounts of Proto IX present inside and outside the chloroplast, intraplastidic and extraplastidic Proto IX pool was estimated in 1 h-light-exposed (75 μmol $m^{-2}$ $s^{-1}$) AF-Na-treated plants. Low light intensity and one h illumination period were chosen as the cellular integrity of herbicide-treated plants remained intact un-

Table I. Impairment of photosynthetic electron transport due to photodynamic damage and its recovery in dark. Control and AF-Na-sprayed cucumber seedlings were incubated in dark for 14 h prior to light treatment (75 μmol $m^{-2}$ $s^{-1}$) for 24–72 h and were subsequently transferred to dark for 24 h. Chl a fluorescence transients of leaves were measured by a pulse amplitude modulated Chl fluorometer (PAM 101, Heinz Walz, Germany) . The values are the average of 5 replicates and ± represents SD.

| Sample | Light treatment | Post-light dark treatment | Initial fluorescence ($F_o$) | Maximum fluorescence ($F_m$) | Variable fluorescence ($F_v$) | $F_v/F_m$ | Inhibition |
|---|---|---|---|---|---|---|---|
| | h | | | | | | % |
| Control | 24 | 0 | 10.35 | 60.00 | 49.65 | 0.83 ± 0.019 | |
| Treated | 24 | 0 | 12.51 | 30.00 | 17.49 | 0.58 ± 0.01 | 30 |
| Control | 24 | 24 | 8.80 | 59.00 | 50.20 | 0.85 ± 0.016 | |
| Treated | 24 | 24 | 9.25 | 39.50 | 30.25 | 0.77 ± 0.024 | 10 |
| Control | 48 | 0 | 9.00 | 62.00 | 53.00 | 0.85 ± 0.014 | |
| Treated | 48 | 0 | 10.50 | 16.33 | 5.83 | 0.36 ± 0.004 | 58 |
| Control | 48 | 24 | 11.00 | 62.75 | 51.75 | 0.82 ± 0.019 | |
| Treated | 48 | 24 | 11.22 | 19.75 | 8.53 | 0.43 ± 0.004 | 48 |
| Control | 72 | 0 | 12.25 | 65.00 | 52.75 | 0.81 ± 0.003 | |
| Treated | 72 | 0 | 13.20 | 15.00 | 1.80 | 0.12 ± 0.011 | 85 |
| Control | 72 | 24 | 9.75 | 62.00 | 52.25 | 0.84 ± 0.017 | |
| Treated | 72 | 24 | 9.20 | 12.30 | 3.10 | 0.25 ± 0.008 | 70 |

der these conditions (Nandihalli *et al.*, 1991). When AF-Na-treated and 14 h-dark-incubated plants were exposed to light for 1 h cotyledons of these plants accumulated 2.6 nmol Proto IX (g Fwt)$^{-1}$. The plastidic and extraplastidic distribution of this 2.6 nmol Proto IX accumulated in 1 g of leaf material was determined as follows.

Chl content of cucumber cotyledons of AF-Na-treated and 1 h-light-exposed plants was 2198 μg (g Fwt)$^{-1}$. To determine the chloroplastic Proto IX content, intact chloroplasts were isolated from AF-Na-treated and 1 h-light-exposed plants and 100 μl aliquot of the intact chloroplast sample was used to determine the Chl content of the chloroplasts, which was found to be 19.62 μg Chl. Another 100 μl aliquot of the intact chloroplast sample was used to determine Proto IX content, which was 0.005 nmol. Thus intact chloroplasts having 19.62 μg Chl contained 0.005 nmol of Proto IX. Chls of plant tissues are exclusively localised in chloroplasts. Simple mathematical calculation revealed that in treated tissue containing 2198 μg Chl (g Fwt)$^{-1}$ had 0.56 nmol of chloroplastic Proto IX. Thus, out of 2.6 nmol of Proto IX present in 1 g of AF-Na-treated tissue, only 0.56 nmol were present inside chloroplasts, which amounted to 22% of the total Proto IX present in the tissue. Rest of the Proto IX (78%) was outside the chloroplasts (Table II). Table II also shows the localisation of protochlorophyllide (Pchlide) within the cell at the end of 14 h dark-incubation of AF-Na-sprayed plants. Tissues were homogenized in dark, and as far as possible isolation of intact chloroplasts was done under safe green light to prevent phototransformation of Pchlide to Chlide. Estimation of intracellular location of Pchlide was done as described for Proto IX. In dark, 82–83% of Pchlide was found to be present within the chloroplast, in both control and treated samples. This was expected since Pchlide is known to be present within chloroplasts. Apparent extraplastidic location of Pchlide (17–18%) was actually a measure

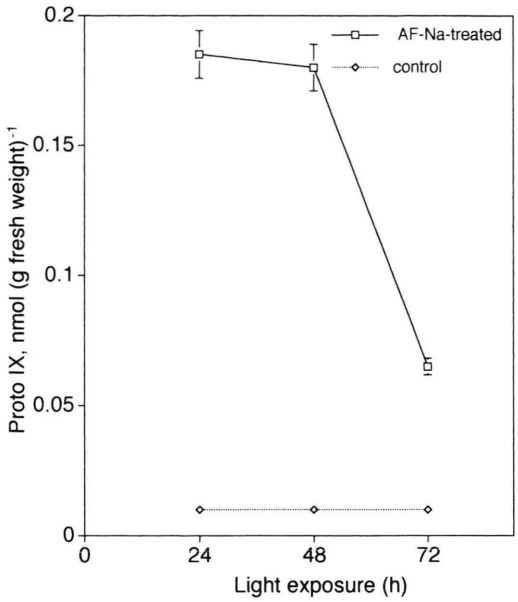


Fig. 2. Proto IX accumulation in AF-Na-treated plants exposed to coolwhite fluorescent light (50 μmol $m^{-2}$ $s^{-1}$). Proto IX content was corrected for decrease in fresh weight in AF-Na-treated plants. Experimental details are as in Materials and Methods. Each observation is the mean of 5 replicates and error bars represent SD. Missing error bars indicate that they are smaller than the the symbol.

of disappearance of Pchlide due to its partial phototransformation to Chlide during isolation of intact chloroplast. The determination of intraplatidic location of Pchlide confirms that the methodology followed to study the intracellular distribution of tetrapyrroles was correct.

*Effect of Proto IX on photochemical reactions of thylakoid membranes*

Proto IX is a photosensitizer that could cause photodynamic damage to the photosynthetic apparatus of plants. In order to ascertain if Proto IX acting as a photosensitizer could inflict damage to the photosynthetic apparatus, thylakoid membranes were illuminated (250 μmol $m^{-2}$ $s^{-1}$) in absence or presence of different concentrations of exogenously-added Proto IX for up to 30 min and the activities of PSII and PSI reactions were monitored.

Incubation of thylakoid membranes with Proto IX (1500 nM) up to 60 min in dark, did not inhibit PSII and PSI reactions demonstrating that Proto IX itself was not an inhibitor of thylakoid membrane functions. When thylakoid membranes were illuminated in absence of Proto IX for up to 30 min, PSII was only marginally affected (3%) (Fig. 3A). This suggests that the low light intensity used to illuminate thylakoid membranes did not affect PSII. However, when thylakoid membranes were illuminated in presence of Proto IX, PSII activity was impaired. When Proto IX concentration was raised from 600 to 1500 nM, inhibition of PSII activity increased from 19% to 56%, after 30 min of illumination. These data demonstrate that Proto IX acted as a photosensitizer and caused photodynamic damage to the thylakoid membranes.

Contrary to their effect on PSII, exogenous addition of 600 and 900 nM Proto IX to thylakoid membranes caused 35% and 15% enhancement of MV-supported PSI activity respectively, after

Table II. Intracellular distribution of tetrapyrroles in the cotyledons of control and AF-Na-treated cucumber plants. Plants were incubated in dark for 14 h and the intracellular distribution of Pchlide was determined at the end of dark period. Intracellular distribution of Proto IX was estimated after illumination of 14 h-dark-incubated plants with cool white fluorescent light (75 μmol $m^{-2}$ $s^{-1}$) for 1 h. Percent of total Proto IX and Pchlide localized in extraplastidic and intraplastidic regions are shown in parentheses. Values are the average of 3 replicates and ± represents SD.

| Light treatment | Control | | Treated | |
|---|---|---|---|---|
| h | Intraplastidic | Extraplastidic | Intraplastidic | Extraplastidic |
| | | nmol (g Fwt)$^{-1}$ | | |
| | | **Proto IX** | | |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0.56 ± 0.07 (22) | 2.04 ± 0.24 (78) |
| | | **PChlide** | | |
| 0 | 11.6 ± 1.5 (83) | 2 ± 0.25 (17) | 17.4 ± 2.0 (83) | 3.6 ± 0.4 (17) |

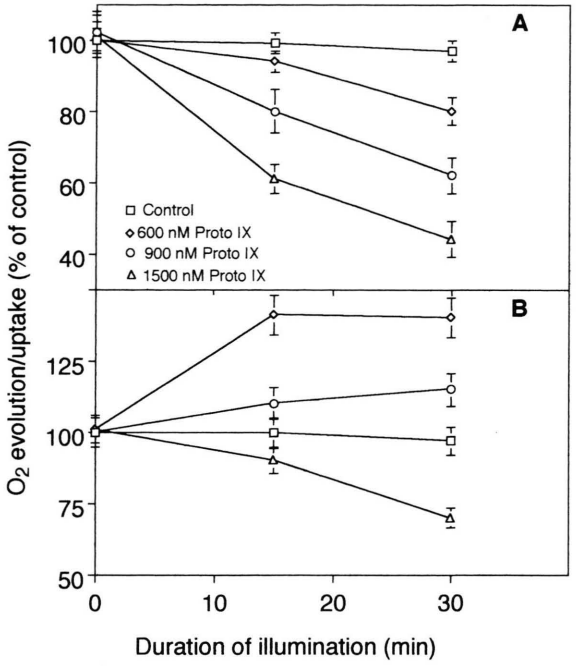


Fig. 3. (A) PSII and (B) PSI activities of isolated thylakoid membranes illuminated in presence of exogenous Proto IX. Thylakoid membranes, suspended at concentration of 1 mg Chl $ml^{-1}$, were incubated without (control) or with different concentrations of exogenous Proto IX and were illuminated with white light from a tungsten light source (250 μmol $m^{-2}$ $s^{-1}$) for desired length of time. PSII activity in control or treated thylakoids before light treatment was 90 ± 10 μmol $O_2$ evolved mg $Chl^{-1}$ $h^{-1}$ and the same for PSI was 200 ± 20 μmol $O_2$ uptake mg $Chl^{-1}$ $h^{-1}$. Other experimental details were as mentioned in Materials and Methods. Each observation is the mean of 5 replicates and error bars represent SD.

30 min of illumination (250 μmol $m^{-2}$ $s^{-1}$) (Fig. 3B). However, when Proto IX concentration was increased to 1500 nM the PSI activity was inhibited by 25%.

To investigate whether Proto IX-mediated photodynamic damage of PSII activity was only due to the impairment of water-splitting-enzyme system, or also resulted from damage of pigment-protein complexes, the effect of exogenous PSII electron donors $Mn^{2+}$, DPC and $NH_2OH$ (Izawa, 1970; Tripathy and Mohanty; 1980; Sharp and Yocum, 1981) on Chl a fluorescence was measured. Illumination (250 μmol $m^{-2}$ $s^{-1}$) of thylakoids (1 mg Chl $ml^{-1}$) in the presence of Proto IX (950 nM) for 30 min, reduced the $F_v/F_m$ by 28% (i.e., from 0.69 in control to 0.5 in treated) (Table III) demonstrating the loss of PSII activity. The exogenous electron donors failed to restore the inhibited $F_v/F_m$ ratio in treated samples to control level. These data suggest that the mechanism of loss of PSII activity was due to the damage to the pigment-protein complex of PSII.

*Effect of scavengers of active-oxygen species on Proto IX-induced photodynamic damage to PSII*

In order to study if Proto IX-induced photodynamic damage to thylakoid membrane was mediated by active-oxygen species, the scavengers of $^1O_2$, $O^{2-}$ and $OH^-$ were applied to the illuminated thylakoid membranes in presence of Proto IX. Due to illumination of thylakoid membranes in the presence of 950 nM Proto IX for 30 min PSII activity was reduced by 55% (Fig. 4). Addition of 20 mM L-histidine (prepared from L-histidine. HCl in 50 mM Hepes/NaOH pH 6.8), a $^1O_2$ scavenger, (Matheson *et al.*, 1975) to the above illuminated reaction mixture protected PSII function in treated thylakoids by 65–70%. Scavengers of $O^{2-}$, superoxide dismutase (SOD) (100 units $ml^{-1}$) (Fridovich, 1978; Henderson and Miller, 1986) and 1,2-dihydroxybenzene-3, 5-disulfonic acid (TIRON) (50 mM) (Miller and Macdowall, 1975; Shimazaki *et al.*, 1980) failed to protect PSII from Proto IX-induced photodynamic damage. The $OH^-$ -scavengers formate (10 mM) (Feierabend and Winkelhusener, 1982) and *tert*-butanol (Czapski, 1984; Ewing and Kubala, 1987) were also unable to protect PSII. These results demonstrate that Proto IX-sensitized photodynamic injury to thylakoid membrane is mediated by $^1O_2$.

*Production of superoxide radical*

Unlike type II photosensitization reactions, in type I photosensitization reactions the triplet sensitizer reacts directly with substrate and $O_2$ to generate $O_2^-$. To investigate if in addition to the production of $^1O_2$, $O_2^-$ was also produced by the type I photosensitization reaction, $O_2^-$ – mediated cytochrome C reduction was investigated using thylakoid membranes, in the absence and presence of DCMU, a photosynthetic electron transport inhibitor (Table IV). Thylakoid membranes were incubated without (control) or with 950 nM Proto IX (treated) and were illuminated (1500 μmol $m^{-2}$ $s^{-1}$) for 1 min. After illumination, the amounts of

Table III. Effect of exogenous PSII electron donors on Chl a fluorescence transients of isolated thylakoid membranes. Thylakoid membranes (1 mg Chl $ml^{-1}$) illuminated with white light (250 μmol $m^{-2}$ $s^{-1}$) for 30 min in the absence (control) or presence (treated) of the photosensitizer Proto IX (950 nM). For the assay of Chl a fluorescence transients thylakoid membranes were suspended at a Chl concentration of 15 μg Chl $ml^{-1}$ and dark-adapted for 15 min. Fluorescence transients were measured in a PAM Chlorophyll fluorometer. Exogenous electron donors were added to the reaction mixture immediately prior to fluorescence measurements. Each value is the average of 5 replicates and ± represents SD.

| Fluorescence transients | No addition | +$NH_2OH$ (10 mM) | +$MnCl_2$ (0.3 mM) | +DPC (2 mM) |
|---|---|---|---|---|
| | | **Control** | | |
| Initial fluorescence ($F_o$) | 3.8 ± 0.21 | 4.0 ± 0.23 | 3.75 ± 0.2 | 4.0 ± 0.23 |
| Maximum fluorescence ($F_m$) | 12.4 ± 0.7 | 11.8 ± 0.7 | 12.4 ± 0.77 | 12.5 ± 0.83 |
| Variable fluorescence ($F_v$) | 8.8 ± 0.5 | 8.3 ± 0.48 | 8.65 ± 0.97 | 8.9 ± 0.85 |
| $F_v/F_m$ | 0.69 ± 0.01 | 0.66 ± 0.01 | 0.69 ± 0.04 | 0.68 ± 0.03 |
| | | **Treated** | | |
| Initial fluorescence ($F_o$) | 4.5 ± 0.23 | 4.6 ± 0.22 | 5.0 ± 0.25 | 5.2 ± 0.22 |
| Maximum fluorescence ($F_m$) | 9.0 ± 0.58 | 9.3 ± 0.65 | 10.0 ± 0.59 | 10.2 ± 0.5 |
| Variable fluorescence ($F_v$) | 5.0 ± 0.4 | 4.7 ± 0.83 | 5.0 ± 0.38 | 5.0 ± 0.71 |
| Fv/Fm | 0.5 ± 0.01 | 0.5 ± 0.05 | 0.5 ± 0.01 | 0.49 ± 0.04 |

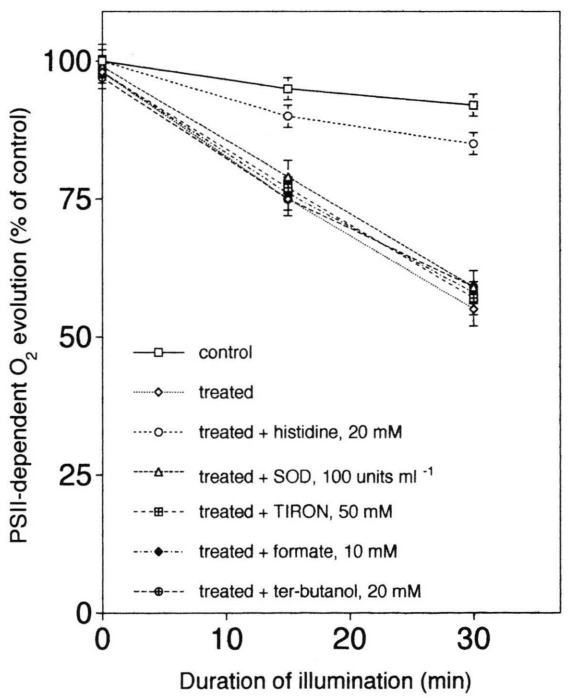


Fig. 4. Effect of scavengers of active oxygen species on PSII activity of isolated thylakoid membranes illuminated in presence of exogenous Proto IX. Thylakoid membranes (1 mg Chl $ml^{-1}$) in absence (control) or presence of 950 nM Proto IX (treated) were illuminated (250 μmol $m^{-2}$ $s^{-1}$) along with scavengers of active oxygen species. PSII activity was assayed after 0, 15 and 30 min of illumination. The control rate of $O_2$ evolution was 95 μmol mg $Chl^{-1}$ $h^{-1}$. Each observation is the mean of 5 replicates and error bars represent SD.

Table IV. Production of $O_2^-$ by isolated thylakoid membranes. $O_2^-$ production was monitored as cytochrome C reduction in isolated thylakoid membranes (50 μg Chl) in the absence (control) or presence (treated) of 950 nM Proto IX. The reaction mixture was illuminated at 1500 μmol $m^{-2}$ $s^{-1}$ for 20 seconds and cytochrome C reduction was monitored in the absence or presence of DCMU at 550 nm. Values are the average of 3 replicates and ± represents SD.

| Sample | Cytochrome C assay | |
|---|---|---|
| | −DCMU | +DCMU |
| | [nmol mg $Chl^{-1}$ $min^{-1}$] | |
| Control thylakoids | 77.4 ± 7.0 | 5.2 ± 0.5 |
| Treated thylakoids | 74.3 ± 6.0 | 6.0 ± 0.6 |

$O_2^-$ produced in control and treated thylakoids were almost equal. In DCMU-poisoned thylakoids, the production of $O_2^-$ was significantly reduced to similar amounts in both control and treated samples. This suggests that production of $O_2^-$ in control and treated thylakoids was not from type I photosensitization reaction of Proto IX, but originated from the photosynthetic electron transport reactions i.e., mostly from PSI and possibly from Rieske iron-sulfur centers.

## Discussion

The loss of Chl and carotenoid contents of AF-Na-treated cucumber plants was less at lower and more at higher light intensity (Fig. 1) suggesting

that generation of active oxygen species involved in photodynamic damage was dependent on light intensity.

Exposure of AF-Na-treated plants to light caused inhibition of PSII function, which was monitored in intact leaves by Chl *a* fluorescence induction kinetics. Illumination of AF-Na-treated plants caused photodynamic injury to plants. This resulted in damage to thylakoid membranes and consequently PSII activity and $F_v/F_m$ ratio declined. After transfer of light-treated (24 h) plants to dark there was substantial recovery from photodynamic damage which resulted in substantial restoration of $F_v/F_m$ ratio. However, when photodynamic damage was more severe after 48–72 h light exposure, dark-recovery from photodynamic damage was only marginal as indicated by slight restoration of the inhibition in $F_v/F_m$ ratio (Table I). This suggests that when initial damage to treated plants was below some threshold (due to shorter duration and lower intensity of light exposure), the repair processes occurring in darkness were more effective. The dark recovery of AF-Na-induced photodynamic damage is interesting. Further investigations are needed to understand the exact mechanism of dark recovery of PSII.

The tetrapyrrole Proto IX acts as the photosensitizer in AF-Na-treated plants (Matringe and Scalla, 1988). AF-Na-treated seedlings over-accumulate Proto IX after they are exposed to light (Fig. 2). However, they do not accumulate Proto IX in dark. The exact mechanism of light-induced accumulation of Proto IX is not known (Nandihalli *et al.*, 1991). It is well known that Proto IX accumulates in plants treated with DPE herbicides. Plastidic Protox is shown to be sensitive to DPEs whereas the plasma membrane bound Protogen IX oxidiser is insensitive to DPEs (Jacobs *et al.*, 1991; Lee *et al.*, 1993; Duke *et al.*, 1994). Incubation of intact plastids with ALA and AF-Na in light caused extraplastidic migration of Protogen IX (Jacobs and Jacobs, 1993). The porphyrin fluorescence was seen both in plastids as well as throughout the cytoplasm in tissues treated with DPE herbicide, while in controls fluorescence was almost exclusively located in plastids (Lehnen *et al.*, 1990). However, these were relative studies and there was no report of quantitative comparison of Proto IX present within plastids and at extraplastidic locations. The present study shows that out of Proto IX that accumulated in the cotyledons of AF-Na-treated and 1 h-light-exposed plants, 22% was located within the chloroplasts and 78% was outside chloroplasts (Table II). This demonstrates that most of Protogen IX that is synthesized in the chloroplast migrate out of the plastid and confirms the hypothesis regarding migration of Protogen IX to extraplastidic locations. Data further suggest that in cucumber cotyledons migration of protogen IX from chloroplast is operating within 1 h of exposure of herbicide-treated plants to light.

Protogen IX synthesis takes place within the chloroplast and compared to none in control around 22% of Proto IX is present within the chloroplasts of treated samples. Therefore, it is likely that chloroplast membranes are the target of photodynamic reactions. As described in results incubation of 1500 nM Proto IX with thylakoid membranes in dark for 1 h did not have any effect on PSI and PSII photochemical reactions which suggested that Proto IX itself did not have any inihibitory effect on thylakoid membrane functions. Illumination of thylakoid membranes in the presence of exogenously added photosensitizer Proto IX caused photodynamic injury to PSII (Fig. 3A). However, increase in PSI activity due to illumination of thylakoid membranes in the presence of 600–900 nM Proto IX was probably due to damage to the PSI localised 18 kD subunit II (*Psa* D gene product) and 11 kD subunit IV (*Psa* E gene product). These proteins act as a docking site for ferredoxin and also shield the PSI reaction center. Removal of the two proteins allows exogenous electron acceptors more access to the reaction center, which could result in the higher activity of MV-supported PSI reaction (Chitnis *et al.*, 1986). Inhibition of PSI reaction observed at 1500 nM Proto IX was probably due to damage to pigment-protein complex associated with the reaction center of PSI (Fig. 3B). These experiments demonstrated that Proto IX acted as a photosensitizer and caused photodynamic damage to the membranes.

Illumination of thylakoid membranes with Proto IX actually resulted in photodynamic damage to PSII. L-histidine, a scavenger of $^1O_2$, protected the PSII pigment-protein complex from Proto IX-mediated photodynamic damage (Fig. 4). However, formate and *tert*-butyl alcohol, scavenger of hydroxyl radical and superoxide dismutase

and tiron, scavengers of superoxide radical, failed to protect PSII activity from Proto IX-mediated photodynamic damage. These results demonstrated that Proto IX-sensitized photodynamic damage of the thylakoid membranes could be protected by scavenging $^1O_2$. In a previous study exogenous application of ALA caused over-accumulation of the photosensitizer Pchlide which produced $^1O_2$ and L-histidine, by scavenging $^1O_2$, protected the thylakoid membrane-linked PSII reaction (Chakraborty and Tripathy, 1992; Tripathy, 1994).

In addition to the type II photosensitization reaction of Proto IX, which generates $^1O_2$, type I photosensitization reaction could lead to the production of $O_2^-$. However, abolition of $O_2^-$ production by DCMU in both control and treated thylakoid membranes (Table IV) indicates that $O_2^-$ was formed at the reducing side of PSI and possibly from Rieske Fe-S centers and not from type I photosensitization reaction of Proto IX.

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Asada K. (1984), Chloroplasts: formation of active oxygen and its scavenging. Methods Enzymol. **105**, 422–429.

Böger P. (1984), Multiple modes of action of diphenyl ethers. Z. Naturforsch. **39c**, 468–475.

Chakraborty N. and Tripathy B. C. (1992), Involvement of singlet oxygen in 5-aminolevulinic acid-induced photodynamic damage of cucumber (*Cucumis sativus* L.) chloroplasts. Plant Physiol. **98**, 7–11.

Chitnis P. R., Reilly P. A. and Nelson N. (1986), Insertional inactivation of the gene encoding subunit II of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. J. Biol. Chem. **264**, 18381–18385.

Czapski G. (1984), On the use of OH· scavengers in biological systems. Isr. J. Chem. **24**, 29–32.

Duke S. O., Becerril J. M., Sherman T. D., Lydon J. and Matsumoto H. (1990), The role of protoporphyrin IX in the mechanism of action of diphenyl ether herbicides. Pestic. Sci. **30**, 367–378.

Duke S. O. and Rebeiz C. A. (1994), Porphyrin biosynthesis as a tool in pest management an overview, in: Porphyric Pesticides Chemistry, Toxicology and Pharmaceutical Applications (S. O. Duke and C. A. Rebeiz eds.) pp.1–16, ACS Symposium Series No.599, Washington D. C.

Ewing D. and Kubala G. J. (1987), Radiation protection of *Escherichia coli* B/r by hydroxyl radical scavengers. Radiat. Res. **109**, 256–274.

Feierabend J. and Winkelhusener T. (1982), Nature of photooxidative events in leaves treated with chlorosis-inducing herbicides. Plant Physiol. **70**, 1277–1282.

Fridovich I. (1978), The biology of oxygen radicals. The superoxide radical is an agent of oxygen toxicity; superoxide dismutase provide an important defense. Science **201**, 875–880.

Henderson B. W. and Miller A. C. (1986), Effects of scavengers of reactive oxygen and radical species on cell survival following photodynamic treatment *in vitro*: Comparison to ionizing radiation. Radiat. Res. **108**, 196–205.

Hukmani P. and Tripathy B. C. (1992), Spectrofluorometric estimation of intermediates of chlorophyll biosynthesis: protoporphyrin IX , Mg-protoporphyrin, and protochlorophyllide. Anal. Biochem. **206**, 125–130.

Izawa S. (1970), Photoreduction of 2,6-dichlorophenol-indophenol by chloroplasts with exogenous $Mn^{2+}$ as electron donor. Biochim. Biophys. Acta **197**, 328–331.

Jacobs J. M., Jacobs N. J., Sherman T. D. and Duke S. O. (1991), Effect of diphenyl ether herbicide on oxidation of protoporphyrinogen to porphyrin in organellar and plasma membrane enriched fractions of barley. Plant Physiol. **97**, 197–203.

Lee H. J., Duke M. V. and Duke S. O. (1993), Cellular localization of protoporphyrinogen- oxidizing activities of etiolated barley (*Hordeum vulgare* L.) leaves. Plant Physiol. **102**, 881–889.

Lehnen I. P., Sherman T. D., Becerril J. M. and Duke S. O. (1990), Tissue and cell localisation of acifluorfen-induced porphyrins in cucumber cotyledons. Pestic. Biochem. Physiol. **37**, 239–248.

Matheson B. C., Etheridge R. D., Kratowich N. R. and Lee J. (1975), The quenching of singlet oxygen by amino acids and proteins. Photochem. Photobiol. **21**, 165–171.

Matringe M. and Scalla R. (1988), Studies on the mode of action of acifluorfen-methyl in nonchlorophyllous soybean cells. Plant Physiol. **86**, 619–622.

Matsumoto H., Lee J. J. and Ishizuka K. (1994), Variation in crop resistance to protoporphyrinogen oxidase inhibitors, in: Porphyric Pesticides Chemistry, Toxicology and Pharmaceutical Applications (S. O. Duke and C. A. Rebeiz eds.). ACS Symposium Series No.599, Washington D. C. pp.120–132.

Miller, R. W. and Macdowall, F. D. H. (1975), The tiron free radical as a sensitive indicator of chloroplastic peroxidation. Biochim. Biophys. Acta **387**, 176–187.

Nandihalli U. B., Liebl R. A. and Rebeiz C. A. (1991), Photodynamic herbicides VIII. Mandatory requirement of light for the induction of protoporphyrin IX accumulation in acifluorfen-treated cucumber. Pestic. Sci. **31**, 9–23.

Porra R. J., Thompson W. A. and Kriedemann P. E. (1989), Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta. **975**, 384–394.

Rebeiz C. A., Montazer-Zouhoor A., Hopen H. J. and Wu S-M. (1984), Photodynamic herbicides: Concept and phenomenology. Enzyme Microb. Technol. **6**, 390–401.

Rebeiz C. A., Montazer-Zouhoor A., Mayasich J. M., Tripathy B. C., Wu S-M. and Rebeiz C. C. (1988), Photodynamic herbicides. Recent development and molecular basis of selectivity. CRC Crit. Rev. Plant Sci. **6**, 385–434.

Schreiber, U. (1986), Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. Photosynth. Res. **9**, 261–272.

Sharp R. R. and Yocum C. F. (1981), Factors influencing hydroxylamine inactivation of photosynthetic water oxidation. Biochim. Biophys. Acta **635**, 90–104.

Shimazaki K., Sakaki T., Kondo N. and Sugahara K. (1980), Active oxygen participation in chlorophyll destruction and lipid peroxidation in $SO_2$ – fumigated leaves of spinach. Plant Cell Physiol. **21**, 1193–1204.

Tewary AK, Tripathy BC (1998), Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber (*Cucumis sativus* L) and wheat (*Triticum aestivum* L). Plant Physiol. **117**, 851–858.

Tripathy B. C and Chakraborty N. (1991), 5-Aminolevulinic acid induced photodynamic damage of the photosynthetic electron transport chain of cucumber (*Cucumis sativas* L.) cotyledons. Plant Physiol. **96**, 761–767.

Tripathy B. C. and Mohanty P. (1980), Zinc-inhibited electron transport of photosynthesis in isolated barley chloroplasts. Plant Physiol. **66**, 1174–1178.

Tripathy B. C. (1994), 5-Aminolevulinic acid induced photodynamic damage to cucumber (*Cucumis sativas* L.) plants mediated by singlet oxygen. In: Porphyric Pesticides Chemistry, Toxicology and Pharmaceutical Applications (S. O. Duke and C. A. Rebeiz eds.). ACS Symposium Series No.599, Washington D. C., pp.65–80.

Wellburn A. R. and Lichtenthaler H. (1984), Formulae and program to determine total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. In: Adv. Photosynth. Res. Vol.II, (C. Sybesma, ed.). Martinus Nijhoff/Dr.W. Junk Publishers, The Hague, pp 9–12.

Witkowski D. A. and Halling B. P. (1988), Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. Plant Physiol. **86**, 632–637.