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BRIEF COMMUNICATION

# Dynamic changes of photosynthetic pigments in soybean callus under high irradiance

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## Abstract

Dynamic changes of neoxanthin (NEO), violaxanthin (VIO), anteraxanthin (ANT), zeaxanthin (ZEA), chlorophyll (Chl) *a,* Chl *b*, α-carotene, β-carotene, and their behaviour under increasing duration of high irradiance (HI) were investigated in the soybean hypocotyl callus culture. The calli were induced on solid (1.1 % agar) MS medium (pH 5.8) supplemented with 4.52 μM 2,4-D, 2.32 μM kinetin, and 3 % sucrose. After 30 d of culture, the green calli were irradiated with "white light" (133 W $m^{-2}$) for 0, 3.5, and 24 h. HPLC profiles were separated on a $C_{18}$ column. With increasing duration of HI, the content of total carotenoids (Cars) increased, but the ratio of Chl *a*+*b*/Cars decreased. With lengthening the duration of HI, there was induction of ZEA. Contents of ANT, α-carotene, and β-carotene remained nearly constant, but ratio of ZEA/Chl *a*+*b* increased with lengthening the HI duration.

*Additional key words*: carotenoids; chlorophyll; *Glycine max*; HPLC; photoinhibition; spectrophotometry; violaxanthin; zeaxanthin.

Under high irradiance (HI) photosynthetic activities are highly susceptible to photoinhibition (Long *et al.* 1994, Osmond 1994). Exposure of leaves to HI reduces photosynthetic efficiency; this phenomenon is called photoinhibition (Powles 1984, Demmig-Adams and Adams 1992). Higher plants and green algae collect photons for photosynthesis by antenna system consisting of pigment-protein complexes. These complexes contain chlorophylls (Chl) *a* and *b,* and a variety of carotenoids (Cars) with different cyclic end-groups (Cunningham and Gantt 1998). The various Cars are distributed among the photosynthetic complexes in thylakoid membrane. They harvest photons, maintain structure and function of the photosynthetic complexes, quench Chl triplet states, and dissipate excess energy (Demmig-Adams *et al*. 1996, Niyogi 1999). The fact that different Cars and Chls are located within different pigment-protein complexes in the photosynthetic apparatus (Siefermann-Harms 1985) makes it highly desirable to have a method for quantifying the amounts of individual pigments (de las Rivas *et al*. 1989). Imposition of HI on high nitrogen treated *Coffea arabica* L. accompanied an increase in energy dissipation mechanisms that include an increase in the 'high energy' quenching and, mostly, the presence of higher contents of some xanthophylls (ZEA and lutein) and carotenes (Ramalho *et al*. 2000). Irradiance also affects pigment contents during transfer of plantlets from *in vitro* to *ex vitro* conditions (Semorádová *et al*. 2002). However, information available for photosynthetic pigments and characteristics with HI on *in vitro* green callus culture of soybean is scanty. Therefore we studied the dynamic changes of various photosynthetic pigments and their behaviour with increasing duration of HI, *i.e*. 0, 3.5, and 24 h in *in vitro* green calli of soybean.

The seeds of soybean (*Glycine max* cv. Pungsannamulkong) from National Honam Agricultural Experiment Station, Rural Development Administration, Iksan, Republic of Korea were sterilised with 70 % ethanol for

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*Abbreviations*: ANT – antheraxanthin; Cars – carotenoids; Chl – chlorophyll; HI – high irradiance; HPLC – high-performance liquid chromatography; NEO – neoxanthin; PS – photosystem; VIO – violaxanthin; ZEA – zeaxanthin.
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1 min and 1 % sodium hypochlorite solution for 20 min and then subsequently were rinsed 4 times with sterilised distilled water. They were allowed to germinate at 30±1 °C in darkness for 5 d on two layers of *Advantec Toyo* (No. *2*) filter paper in 100 $cm^3$ beakers containing 10 $cm^3$ of distilled water that had been sterilised for 20 min at 121 °C. The whole etiolated excised hypocotyls (0.2–0.3 cm in length) from the 5 d seedlings were cultured for one month in a 100 $cm^3$ flask containing 25 $cm^3$ of solid MS medium (Murashige and Skoog 1962) supplemented with 4.52 μM 2,4-D, 2.32 μM kinetin, 3 % sucrose, and 1.1 % agar. The medium pH 5.8 was adjusted with 1 M KOH. The medium was autoclaved at 121 °C for 20 min. Culture conditions included "white fluorescent light" (15 μmol $m^{-2}$ $s^{-1}$) and an incubator (25±1 °C). After 30 d, the green calli with the thickness of 0.4–0.6 cm were irradiated with "white light" from 150 W *HQI* lamp with irradiance of 133 W $m^{-2}$ for 0, 3.5, and 24 h.

Photosynthetic pigments were extracted by homogenisation of 2 g of soybean calli in a crucible mortar with quartz sand. Sodium carbonate (0.5 g) was added to minimise acid-catalysed isomerisation of Cars. Following homogenisation, 4 $cm^3$ of acetone was added and macerate was again homogenised with 2 $cm^3$ of acetone. The two fractions were collected and centrifuged with a *Beckman J2-MC* centrifuge (USA) at 12 100×*g* for 20 min. The supernatant was kept in the dark at 4 °C. Absorbances at 470, 646, and 663 nm were recorded with *Ultrospec 3000* UV/Visible Spectrophotometer (*Pharmacia Biotech (Biochrom)*, Cambridge, England). According to the method of Wellburn (1994), the amounts of Chl *a,* Chl *b,* and total Cars in the acetone supernatant were calculated. Another determination of photosynthetic pigments was made with the HPLC, a *Water* series model *206* of liquid chromatograph consisting of a model *510* solvent delivery system, automated gradient controller, and model *486* tunable absorbance detector. A model *740* data modulator recorded the detector signals. The absorbances of Chl *a*, Chl *b*, and Cars were recorded at 450 nm. Separation of photosynthetic pigments was performed on column (30 cm×3.9 mm) packed with *μBondapak*$^{TM}$ $C_{18}$ particles. According to the de las Rivas *et al*. (1989) method, two mobile phases were used for separation: (I) acetonitrile : methanol (7 : 1, v/v) and (II) acetonitrile : methanol : water : ethyl acetate (7.00 : 0.96 : 0.04 : 2.00, v/v). Solvents were HPLC and/or ACS-ISO grade. Salts were reagent grade. Water was purified with *Puris Ultrapure* Water System, model *MR-UP900* (Republic of Korea). An injection volume was 0.02 $cm^3$ with the flow rate of 1.0 $cm^3$ $min^{-1}$ for the period of 15 min to separate the pigments. The method was calibrated using isolated pigments the concentrations of which were determined according to Val *et al*. (1986). Peak identification for various photosynthetic pigments was done by injecting known amounts of pure pigments, and plotting peak area *versus* mass of the pigment injected (de las Rivas *et al*. 1989).

Using 30-d soybean calli, the amounts of Chl *a*, Chl *b,* total Chl, Cars, and total photosynthetic pigments increased after 3.5-h HI treatment. However, with lengthening the duration of HI (24 h), the amounts of Chl *a* and Chl *b* decreased ($p < 0.01$). The ratio of Chl *a*/*b* declined a little after 3.5 h HI, but increased with lengthening the HI ($p < 0.01$). The amounts of total Cars increased, while ratio of total Chl/Cars decreased (Table 1).

Table 1. Spectral determination of amounts of photosynthetic pigments [mg $kg^{-1}$(f.m.)] in soybean hypocotyl calli under high irradiance (HI) and relative pigment quantities [%] determined by HPLC using mobile phase II, *i.e*. in acetonitrile : methanol : water : ethyl acetate (7.00 : 0.96 : 0.04 : 2.00, v/v) on a $C_{18}$ columns with flow rate of 1.0 $cm^3$ $min^{-1}$ and the injection volume of 0.02 $cm^3$. For details see the text. Means±SE of 5 replications.

| Pigment | Duration of HI treatment [h] | | |
|---|---|---|---|
| | 0 | 3.5 | 24.0 |
| | [mg $kg^{-1}$(f.m.)] | | |
| Chl *a* | 9.42±0.02 | 12.32±0.01 | 10.09±0.05 |
| Chl *b* | 2.93±0.02 | 3.96±0.01 | 3.00±0.10 |
| Chl *a*/*b* | 3.22 | 3.11 | 3.36 |
| Chl *a*+*b* | 12.35±0.03 | 16.28±0.01 | 13.09±0.15 |
| Carotenoids (Cars) | 4.50±0.01 | 6.00±0.01 | 6.04±0.00 |
| Total pigments | 16.85±0.03 | 22.28±0.01 | 19.13±0.15 |
| Chls/Cars | 2.74 | 2.71 | 2.17 |
| | [%] | | |
| NEO | 8.1 | 5.0 | 7.5 |
| VIO | 16.7 | 10.8 | 12.9 |
| ANT | 9.1 | 10.5 | 9.5 |
| ZEA | 16.9 | 26.9 | 29.1 |
| Chl *a* | 33.0 | 31.1 | 26.7 |
| Chl *b* | 5.9 | 5.3 | 4.8 |
| α-carotene | 3.9 | 4.2 | 3.8 |
| β-carotene | 6.4 | 6.2 | 5.7 |
| Total Cars | 61.1 | 63.6 | 68.5 |
| Chl *a*+*b*/total Cars | 0.64 | 0.57 | 0.46 |
| ZEA/Chl *a*+*b* | 0.43 | 0.74 | 0.92 |

With the mobile phase II, we achieved the separation of the full profiles of photosynthetic pigments, *i.e*. neoxanthin (NEO), violaxanthin (VIO), anteraxanthin (ANT), zeaxanthin (ZEA), Chl *a*, Chl *b*, α-carotene, and β-carotene in 15 min. After 3.5-h HI, contents of NEO and VIO decreased and the content of ZEA was 59 % higher. This ZEA induction was further increased to 72 % after 24-h HI treatment. Contents of ANT, α-carotene, and β-carotene remained nearly constant, but the contents of Chl *a* and Chl *b* decreased with lengthening the HI (Fig. 1). The ratio of ZEA/Chl *a*+*b* increased with lengthening the duration of HI (Table 1). The content of total Cars increased with length of HI treatment. Thus short duration of HI enhances the process of pigment

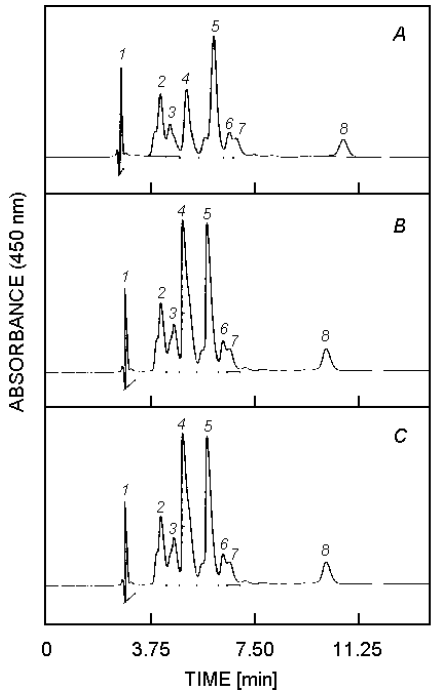


Fig. 1. HPLC profiles of photosynthetic pigments in the soybean calli affected by high irradiance for 0 h (*A*), 3.5 h (*B*), and 24 h (*C*). For details of analysis, see the text. The identified photosynthetic pigments were neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, Chl *a*, Chl *b*, α-carotene, and β-carotene.

synthesis, however, longer duration of HI starts deterioration of the pigment synthesis. This is in agreement with findings of Demmig *et al*. (1987, 1988), Demmig-Adams and Adams (1992), and Powles (1984).

Koroleva *et al*. (2000) described that in *in vivo Lycopersicum esculentum* and *L. peruvianum* leaves at low temperature higher contents of ZEA and ANT and higher ZEA/total Chl ratios were formed under HI. The enhanced thermal energy conversion associated with increased ZEA and ANT contents may protect *in vivo* leaves from severe photodestruction during irradiance stress. According to Ramalho *et al*. (2000), imposition of HI on high nitrogen-treated leaves of *Coffea arabica* accompanied an increase in energy dissipation mechanisms that include an increase in the 'high energy' quenching and, mostly, the presence of higher contents of some xanthophylls (ZEA and lutein) and carotenes. This helps to decrease the energetic overcharges in PS1 and PS2.

With mobile phase I, we did not find any peak for ZEA, α-carotene, and β-carotene (data not shown). In our experiments, lutein was not separated, probably due to its small quantity in the extracts. Similar to our results, Minguez-Mosquera and Hornero-Mendéz (1993) were unable to separate lutein from ripe red pepper leaves. However, de las Rivas *et al*. (1989) described in *Populus nigra* leaves a proper separation of lutein at 4.1-min retention.

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