Effects of different irradiances on the photosynthetic process during *ex-vitro* acclimation of *Anoectochilus* plantlets

D.M. PANDEY[*,**], K.W. YU[*], R.Z. WU[*], E.-J. HAHN[*], and K.-Y. PAEK[*,***]

Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheong-ju 361-763, South Korea[*]
Plant Molecular Biology Laboratory (Functional Genomics Lab), Department of Life Science, Sogang University, Seoul, South Korea[**]

Abstract

Six months old *in vitro*-grown *Anoectochilus formosanus* plantlets were transferred to *ex-vitro* acclimation under low irradiance, LI [60 μmol(photon) m^{-2} s^{-1}], intermediate irradiance, II [180 μmol(photon) m^{-2} s^{-1}], and high irradiance, HI [300 μmol(photon) m^{-2} s^{-1}] for 30 d. Imposition of II led to a significant increase of chlorophyll (Chl) *b* content, rates of net photosynthesis (P_N) and transpiration (E), stomatal conductance (g_s), electron transfer rate (ETR), quantum yield of electron transport from water through photosystem 2 (Φ_{PS2}), and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, EC 4.1.1.39). This indicates that *Anoectochilus* was better acclimated at II compared to LI treatment. On the other hand, HI acclimation led to a significant reduction of Chl *a* and *b*, P_N, E, g_s, photochemical quenching, dark-adapted quantum efficiency of open PS2 centres (F_v/F_m), probability of an absorbed photon reaching an open PS2 reaction centre (F_v'/F_m'), ETR, Φ_{PS2}, and energy efficiency of CO_2 fixation (Φ_{CO2}/Φ_{PS2}). This indicates that HI treatment considerably exceeded the photo-protective capacity and *Anoectochilus* suffered HI induced damage to the photosynthetic apparatus. Imposition of HI significantly increased the contents of antheraxanthin and zeaxanthin (ZEA), non-photochemical quenching, and conversion of violaxanthin to ZEA. Thus *Anoectochilus* modifies its system to dissipate excess excitation energy and to protect the photosynthetic machinery.

Additional key words: chlorophyll *a* fluorescence; electron transfer rate; photoinhibition; photosystem 2; ribulose-1,5-bisphosphate carboxylase/oxygenase; stomatal conductance; transpiration; xanthophylls.

Introduction

In vitro propagation is an efficient method to produce large amount of uniform plantlets. However, micro-propagated plantlets are associated with several physiological and anatomical abnormalities during *in vitro* growth such as low photosynthesis, non-proper functioning of stomata, malfunctioning of water housekeeping systems mainly due to high humidity inside the culture vessel (Grout and Aston 1978, Kozai 1991). Once transferred to *ex-vitro*, micro-propagated plantlets are (easily) susceptible to photoinhibition because of lack of well-developed physiological systems mentioned above. Therefore, acclimation of micro-propagated plantlets to *ex-vitro* is

Received 5 September 2005, *accepted* 5 January 2006.
[***]Author for correspondence; fax: +82 43 272 5369. e-mail: paekky@chungbuk.ac.kr
Abbreviations: ANT – antheraxanthin; Chl – chlorophyll; *cis*-LUT – *cis*-lutein; DEI – de-epoxidation index; DTT – dithiothreitol; E – transpiration rate; ETR – electron transfer rate; F_m – maximal Chl *a* fluorescence yield recorded during a short pulse of very high irradiance (less than 1 s and several thousands of μmol m^{-2} s^{-1}); F_0 – minimal Chl *a* fluorescence yield recorded at very low irradiance (less than 1 μmol m^{-2} s^{-1}); F_m' – maximal Chl fluorescence in light-adapted state; F_0' – minimal Chl fluorescence in light-adapted state; F_v – variable Chl fluorescence in dark-adapted state; F_v' – variable Chl fluorescence in light-adapted state, F_v/F_m – quantum efficiency of open photosystem 2 centres; FM – fresh mass; g_s – stomatal conductance; G-6-P – glucose-6-phosphate; HI – high irradiance; II – intermediate irradiance; LI, low irradiance; MCE – 2-mercaptoethanol; NPQ, non-photochemical quenching; P_N – net photosynthetic rate; PPF – photosynthetic photon flux; PS2 – photosystem 2; PVP – polyvinylpyrrolidon; q_p – coefficient of photochemical quenching; RuBP – ribulose-1,5-bisphosphate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); TEMED – N,N,N',N'-tetramethylenediamine; VIO – violaxanthin; XC – xanthophyll cycle; ZE – zeaxanthin epoxidase; ZEA – zeaxanthin; Φ_{CO2} – quantum yield to CO_2 fixation; Φ_{PS2} – quantum yield of electron transport from water through PS2.
Acknowledgements: This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for the Development of Advanced Horticultural Technology at Chungbuk National University, Cheong-ju 361-763, South Korea.

a crucial step to cope with the new environment for better growth and development. These environmental changes associated with *in vitro* plantlets can be improved in *ex vitro* by controlling the irradiance (Amâncio *et al*. 1999). Powles (1984) reported that in natural environment plants are exposed to a range of photosynthetic photon fluxes (PPF) that can lead to depression in photosynthetic efficiency (photoinhibition) mainly due to oxidative damage to the photosystem 2 (PS2).

Oxygenic photosynthetic organisms have evolved multiple photo-protective mechanisms to cope with the potentially damaging effects of irradiance, drought, and oxidative stress. The xanthophyll cycle (XC) is one of the main photo-protective mechanisms in photosynthesizing higher plant cells (Demmig-Adams and Adams 1990, Pfündel and Bilger 1994). Zeaxanthin (ZEA) is involved in the de-excitation of excess energy *via* non-radiative dissipation in the pigment bed. This process is associated with the de-epoxidation of violaxanthin (VIO) to ZEA and with the development of a trans-thylakoidal ΔpH. ZEA, and perhaps the intermediate antheraxanthin (ANT), may be responsible for the development of non-radiative energy dissipation (Demmig-Adams and Adams 1992). Epoxidation by XC protects the photosynthetic apparatus by several mechanisms (Havaux and Niyogi 1999), and regulations of the genes involved in xanthophyll biosynthesis are known (Woitsch and Römer 2003).

Anoectochilus formosanus Hayata is a native perennial and terrestrial orchid plant grown in the forests of Taiwan for their beauty because its leaves have network of colourful venation (Jewel orchid). *Anoectochilus* belongs to Orchidaceae and has a great value in herbal medicines. *A. formosanu*s has Crassulacean acid metabolism (CAM) type of carbon fixation (Chang *et al*. 2001). However, our unpublished data indicated that *in vitro* cultured *Anoectochilus* exhibited C_3 type of carbon fixation. *Anoectochilus* orchids are very sensitive to irradiance, relative humidity, and air temperature. However, detailed information on the irradiance requirements on photosynthetic ability, when the plantlets are transferred to *ex-vitro* acclimation from *in vitro* in *Anoectochilus*, is missing. Therefore, we investigated the different irradiance effects on the photosynthetic process during *ex vitro* acclimation of *Anoectochilus* plantlets.

Materials and methods

Plants: Plantlets of *Anoectochilus formosanus* Hayata were propagated according to the methods described in Pandey *et al*. (2006). Six-month-old *in vitro* cultured plantlets (5.4 g fresh mass, FM, and 3.9 leaves on average) were transplanted in conical plastic pots (12×9×15 cm, *Cheong Wun-5*, South Korea) filled with coconut chips. Pots were placed in a culture room under low irradiance, LI [60 μmol(photon) m^{-2} s^{-1}], intermediate irradiance, II [180 μmol(photon) m^{-2} s^{-1}], and high irradiance, HI [300 μmol(photon) m^{-2} s^{-1}] from a *400 W PHILIPS-SON-T* lamp (*Philips*, Belgium) with 14/10 h photoperiod for 30-d acclimation. Day/night temperatures and relative humidity were 25/20 °C and 70–80 %, respectively. Plantlets were watered once a week and fertilized once a month. The nutrient solution contained NO_3-N, NH_4-N, P, K, Ca, and Mg at 8.25, 0.50, 3.75, 6.25, 5.00, and 2.50 mg m^{-3}, respectively, as macronutrients. Micronutrients were composed of Fe, Mn, Zn, B, Cu, and Mo at 0.45, 0.55, 0.26, 0.22, 0.03, and 0.03 mg m^{-3}, respectively. The pH and the EC of the nutrient solution were adjusted and maintained at 6.0 and 1.2 dS m^{-1} using a pH and EC controller (*HM-20E* and *CM-20E*, *TOA*, Tokyo, Japan).

Pigments: The extraction, separation, and quantification of photosynthetic pigments were done according to Pandey *et al*. (2005) and de las Rivas *et al*. (1989). *Anoectochilus* leaf sample was homogenized in acetone and centrifuged at 14 000×*g* and 4 °C for 20 min. Chromatography was carried out using an HPLC system (*Waters 2690* separation module; *Waters 996* photodiode array detector; *Waters* millenium *2010* chromatography manager) on a 100×8 mm *Waters Novapak* C_{18} column (4-μm particle size). Required sample (0.02 cm^3) was injected, and flow rate of the mobile phases were 2 cm^3 min^{-1} and the absorbance was detected at 450 nm. Equilibration of the column was done prior to injecting each sample by flushing with acetonitrile : methanol (7 : 1, v/v) for 7 min. The sample was injected into column and mobile phase was pumped for another 2 min. A mixture of acetonitrile : methanol : water : ethyl acetate (7.00 : 0.96 : 0.04 : 2.00, v/v) was further pumped for 1 min. Finally, a similar solvent mixture, but with increased part of ethyl acetate (8.00), was pumped for 7 min.

Gas exchange and chlorophyll (Chl) *a* fluorescence: After 30 d of the irradiance treatments, *Anoectochilus* leaves were used for the gas exchange studies using a portable infra red gas analyzer (*LI-6400, LI-COR*, Lincoln, NE, USA) coupled with a leaf chamber fluorometer (*LI-6400*) in the mode of simultaneous gas exchange and fluorescence measurements. Gas exchange and Chl fluorescence parameters were recorded on the second leaf under 400 μmol mol^{-1} of CO_2 supplied at a flow rate of 400 μmol s^{-1}, 60–70 % relative humidity, and 25±2 °C leaf temperature. The protocol and Chl *a* fluorescence terminology was similar to that described elsewhere (Genty *et al*. 1989, Kornyeyev *et al*. 2003, Slesak *et al*. 2003).

RuBPCO: Enzyme extraction was made according to Borland *et al*. (1998) with some modifications. *Anoectochilus* leaves (1 g FM) were frozen and homogenized in

liquid nitrogen and dissolved in 4 cm^3 extraction buffer [100 cm^3 of 50 mM Tris-HCl (pH 7.5) containing polyvinylpyrrolidon (PVP, 16 mg), 1 mM dithiothreitol, 2 mM EDTA, 5 mM 2-mercaptoethanol, 2 % PEG 20 000, 20 mM $MgCl_2 \times 6\ H_2O$, 5 mM $NaHCO_3$] at 4 °C. Homogenate was centrifuged at 14 000×*g* and 4 °C for 20 min. Protein concentration was estimated by the method of Bradford (1976) using protein assay dye from *BIO-RAD*. A change in absorbance at 340 nm and 25 °C for 3 min was measured by a spectrophotometer (*UV-1650PC, UV-VISIBLE*, *Shimadzu*, Japan). RuBPCO activity was measured according to Borland *et al*. (1998) with some modifications. The 0.95 cm^3 reaction mixture contained 100 mM Bicine-KOH (pH 8.0), 20 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM ATP, 3.5 mM P-creatine, 0.4 mM NADH, 4 units of creatine-P-kinase, 4 units of glyceraldehyde-3-P-dehydrogenase, 4 units of 3-phosphoglyceric phosphokinase, and 0.5 mM RuBP. The reaction was initiated by adding 0.05 cm^3 of crude extract.

Statistical analysis: Each parameter represents the three independent experiments. Means and standard error were calculated from three replicates. Significant differences between treatment means were determined with Duncan's multiple range test (DMRT) calculated at 5 % level.

Results

Changes in photosynthetic pigments (Table 1) were more remarkable at HI than at II treatment. Plantlets acclimatized at II had significantly higher content of Chl *b* compared to LI treatment. Significant reduction was found in contents of VIO, Chl *a*, Chl *b*, and β-carotene (31, 10, 15, and 26 %, respectively) while increase in contents of ANT, ZEA, and *cis*-lutein (*cis*-LUT) (46.7 %, 3.7 times, and 18.8 %, respectively) was recorded in HI-treated leaves. Content of *cis*-LUT was similar in all irradiance treatments and was taken as an internal standard to quantify the different pigments. The ratio of Chl *a*/*b* remained nearly constant and α-carotene was absent. Depending upon the changes in the contents of xanthophyll cycle pigments (XCP), de-epoxidation index

Table 1. Contents of photosynthetic pigments [μmol m^{-2}], net photosynthetic rate, P_N [μmol m^{-2} s^{-1}], stomatal conductance, g_s [mol m^{-2} s^{-1}], transpiration rate, E [mmol m^{-2} s^{-1}], F_0, F_v/F_m, F_o', F_v'/F_m', q_p, $(1 - q_p)$ F_v'/F_m', NPQ, $(1 - q_p)$/NPQ, ETR, Φ_{PS2}, Φ_{CO2}, and Φ_{CO2}/Φ_{PS2} in daylight of *A. formosanus* under different irradiances. Means ± S.E. ($n = 3$). Means denoted by the same letter did not differ significantly at Duncan's multiple range test (DMRT) calculated at 5 % level.

Parameter	Irradiance [μmol m^{-2} s^{-1}]		
	60	180	300
Neoxanthin	11.4±0.6a	11.3±0.4a	11.0±0.8a
Violaxanthin	25.2±1.5a	25.0±0.6a	17.5±0.9b
Antheraxanthin	3.0±0.3b	3.1±0.3b	4.4±0.5a
Lutein	29.7±1.0a	29.6±1.3a	29.2±2.1a
Zeaxanthin	2.4±0.3b	2.6±0.2b	8.9±0.4a
cis-lutein	3.2±0.4b	2.9±0.2b	3.8±0.5a
Chlorophyll *b*	51.3±2.4b	54.0±1.8a	43.7±2.2c
Chlorophyll *a*	216.0±25.4a	227.0±15.1a	194.0±21.0b
Chlorophyll *a*/*b*	4.21±0.34a	4.20±0.14a	4.44±0.29a
β-carotene	28.5±1.4a	28.2±1.1a	21.1±1.7b
De-epoxidation index	0.18±0.04b	0.19±0.04b	0.43±0.05a
P_N	4.21 ± 0.02b	6.62 ± 1.15a	3.53 ± 0.54c
E	0.21 ±0.00b	0.40 ± 0.01a	0.11 ± 0.00c
g_s	1.22 ±0.01b	1.80 ± 0.04a	0.98 ± 0.02c
F_0	763.1 ± 15.6b	794.4 ± 7.8b	920.6 ± 7.5a
F_0'	632.2 ± 5.9b	662.5 ± 9.0b	790.2 ± 4.0a
F_v/F_m	0.50 ± 0.00a	0.49 ± 0.01a	0.36 ± 0.01b
F_v'/F_m'	0.41 ± 0.01a	0.40 ± 0.01a	0.27 ± 0.01b
q_p	0.22 ± 0.03a	0.24 ± 0.02a	0.11 ± 0.01b
NPQ	0.67 ± 0.07b	0.64 ± 0.03b	0.87 ± 0.04a
$(1 - q_p)$/NPQ	1.16 ± 0.06a	1.19 ± 0.01a	1.02 ± 0.02b
$(1 - q_p)$ F_v'/F_m'	0.32 ± 0.02	0.30 ± 0.01a	0.24 ± 0.00b
ETR	1.30 ± 0.07c	6.20 ± 0.17a	2.55 ± 0.08b
Φ_{PS2}	0.05 ± 0.00b	0.09 ± 0.01a	0.02 ± 0.00c
Φ_{CO2}	0.08 ± 0.01a	0.05 ± 0.00b	0.01 ± 0.00c
Φ_{CO2}/Φ_{PS2}	1.58 ± 0.11a	0.56 ± 0.02b	0.48 ± 0.04c

[DEI = (ANT+ZEA)/(VIO+ANT+ZEA)], *i.e*. the activity of VIO de-epoxidase, was calculated. Compared to LI treatment, DEI remained constant at II while it increased significantly (2.4 times) during HI acclimation.

Changes in gas exchange and Chl fluorescence: Imposition of II led to a significant increase in net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) by 57, 100, and 48 %, respectively, compared to LI acclimated leaves (Table 1). Acclimation during HI resulted in a significant reduction of P_N, E, and g_s by 16, 50, and 20 %, respectively, compared to LI treatment. During II acclimation, values of dark- and light-adapted ground fluorescence (F_0 and F_0', respectively) were similar to those in the LI treatment. On the other hand, HI acclimation resulted in a significant increase in F_0 and F_0' (21 and 25 %, respectively) compared to LI treatment. Moreover, during II acclimation the maximum quantum efficiency of open PS2 centre measured by Chl fluorescence ratio in dark-adapted state (F_v/F_m), probability of an absorbed photon reaching an open PS2 reaction centre (F_v'/F_m'), photochemical quenching (q_p), and non-photochemical quenching (NPQ) remained unchanged compared to LI treated leaves. In contrast, HI acclimation resulted in a significant decrease in F_v/F_m, F_v'/F_m', and q_p (28, 34, and 50 %, respectively) while an increase (30 %) occurred in the NPQ. Derived indices $(1 – q_p)$/NPQ and $(1 – q_p)$ F_v'/F_m' that are used to estimate the susceptibility of PS2 to photoinhibition, remained unchanged at II but decreased significantly (by 12 and 25 %, respectively) during HI acclimation. Although all leaves absorbed a certain fraction of the incident photons, we found differences in pigment composition among treatments. This indicates that absorbance among all the treatments was not similar. Therefore, approximations of the electron transport rate (ETR) through PS2 were calculated according to Genty *et al*. (1989) by multiplying the quantum efficiency of PS2 by the incident photon flux density and an average factor of 0.8 for leaf absorbance, and dividing by a factor 2 to account for the sharing of absorbed photons between the two photosystems. During II acclimation, ETR increased significantly compared to LI. Further, acclimation under HI led to significant reduction of ETR compared to II but remained higher than LI treatment. Quantum yield of electron transport from water through PS2, *i.e*. the actual PS2 efficiency (Φ_{PS2}), increased (80 %) while quantum yield to CO_2 fixation corrected for leaf absorption (Φ_{CO2}) decreased (38 %) significantly at II compared to LI acclimation. However, a significant decrease (60 and 88 %, respectively) in their values was recorded during HI acclimation. The ratio Φ_{CO2}/Φ_{PS2}, which is a measure of the energy efficiency of CO_2 fixation, decreased significantly at II and HI acclimation (65 and 70 %, respectively).

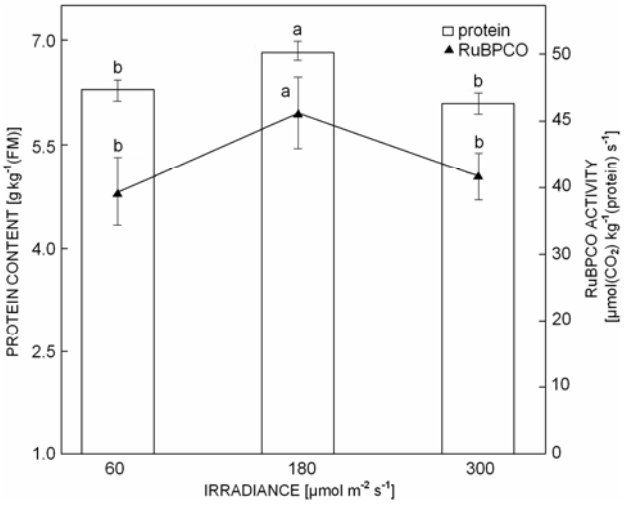

Fig. 1. Activity of ribulose 1,5-bisphosphate carboxylase/oxygenase**,** RuBPCO and content of proteins [g kg^{-1}(FM)] measured in daylight under different iradiances. Means ± S.E. (n = 3). Means denoted by the same letter did not differ significantly at Duncan's multiple range test (DMRT) calculated at 5 % level.

Changes in RuBPCO activity and protein content (Fig. 1): RuBPCO activity increased (29 %) significantly during II acclimation while it decreased at HI treatment compared to LI treatment. Similarly, protein content increased significantly at II and decreased in HI treatment compared to LI-acclimatized plantlets.

Discussion

Plantlets acclimated at II showed slightly higher contents of Chl *a* and *b* (Table 1) indicating their increased synthesis. During II acclimation higher g_s resulted in increased P_N and E that is consistent with the increase in the values of ETR and Φ_{PS2}. However, a significant decrease in the energetic efficiency of CO_2 fixation (Φ_{CO2}/Φ_{PS2}) indicated that during II acclimation the number of electrons required for the fixation per molecule CO_2 decreased significantly (Table 1). Piel *et al*. (2002) reported that higher P_N under HI is associated with increased CO_2 conductance to carboxylation site. Since in C_3 plants RuBPCO is the key enzyme that fixes CO_2, RuBPCO activity was stimulated under II that resulted in higher 3-PGA content. Thus we suggest that II acclimation leads to increase in g_s and RuBPCO activity that result in higher P_N (Table 1, Fig. 1). Compared to LI, higher protein content during II acclimation might be the result of greater protein synthesis (Fig. 1). Similarly, Bertamini and Nedunchezhian (2001) reported that thylakoid membrane protein and RuBPCO activity in full-sunlight [1 500 μmol(photon) m^{-2} s^{-1}] grown grapevine (*Vitis vinifera* L.) leaves increased by 37 and 41 %,

respectively, compared with shaded grapevine [80 μmol (photon) m^{-2} s^{-1}] leaves. Our results indicated that *Anoectochilus* was better acclimatized at II compared to LI.

On the other hand, plantlets acclimated at HI suffer due to excess photons and hence degradation in photosynthetic machinery and/or reduction of antenna size and thereby reduction in the contents of Chl *a* and *b* was recorded (Table 1). In contrast, de las Rivas *et al*. (1989) reported that the 3.5 h-high irradiance treatment of *Populus nigra* leaves resulted in no changes in Chl *a* and *b*. In our study Chl *a*/*b* remained nearly constant. Thus inter-conversion of Chl *a* and Chl *b* is significant for the establishment of required Chl *a*/*b* ratio during the adaptation of leaves to high and low irradiance (Ito *et al*. 1993). During HI acclimation, g_s, E, and P_N decreased significantly and HI-induced photoinhibition was observed that resulted in the reduction of plastoquinone pool (decrease of q_p), F_v/F_m, F_v'/F_m', Φ_{PS2}, Φ_{CO2}, and Φ_{CO2}/Φ_{PS2} (Table 1). Since F_v/F_m was measured after 30 min of dark adaptation, this may have reduced the contribution of energy-dependent non-photochemical quenching to F_v/F_m measurements suggesting that the decline in F_v/F_m represents the accumulation of photo-damaged PS2 centres (Rosenqvist and van Kooten 2003). The reduction of the F_v/F_m ratio observed in HI leaves confirmed the primary target of HI to be PS2 photochemistry. Krause (1988) reported that a decline in F_v/F_m accompanied by an increase in F_0 might indicate that the HI treatment considerably exceeded the photo-protective capacity and resulted in significant photon damage. Since Φ_{PS2} corresponds to P_N, it decreased during HI acclimation. In contrast, compared to LI, HI acclimation led to significant increase of ETR while decrease in P_N and Φ_{CO2}/Φ_{PS2} suggests that electrons were being used in pathways other than CO_2 fixation (Fryer *et al*. 1998). Previously found similar results indicate that short-term HI exposure results in increase in F_0 and decrease in F_v/F_m, Φ_{PS2}, and q_p in a leaf as well as in leaf discs (Slesak *et al*. 2003), decline in F_v'/F_m' (Hymus *et al*. 1999), but an increase in $(1 - q_p)$ F_v'/F_m' (Kornyeyev *et al*. 2003). Pell *et al*. (1994) reported that increase in $(1 - q_p)$ observed during HI acclimation indicated that primary target are the enzymes involved in the Calvin cycle and in particular RuBPCO. The inhibition of RuBPCO induces a lower requirement of NADPH and ATP and this may in turn cause a reduced ETR and an increase in the proportion of closed centres of PS2. Decrease in RuBPCO activity during HI treatment should be attributed to (*1*) HI-induced degradation of photosynthetic machinery, (*2*) conformational modification of enzyme, and (*3*) availability of substrate and other cofactors which are modulated during HI acclimation. Lower protein content during HI acclimation might be the result of increased degradation (Fig. 1). This observation suggests that HI treatment considerably exceeded the photoprotective capacity and *Anoectochilus* suffers HI induced damage to the photosynthetic apparatus.

Imposition of HI significantly increases the contents of ANT and ZEA and enhanced significant conversion of VIO to ZEA. Activity of VIO de-epoxidase (DEI) remained unchanged during II acclimation while increased significantly at HI (Table 1). Similar result was reported previously (Pandey *et al*. 2005). Ramalho *et al*. (2000) reported that imposition of high irradiance resulted in an increase in energy dissipation mechanisms such as 'high energy' quenching and an increase in the contents of ZEA and LUT. Woitsch and Römer (2003) reported that high irradiance activated the mRNAs of VDE that stimulated the conversion of VIO to ZEA, and zeaxanthin epoxidase (ZE) that stimulated the conversion of ZEA into ANT and VIO. In the present study high irradiance-stimulation of endogenous VDE has overcome the endogenous ZE effects. Imposition of HI led to the significant increase in the NPQ, which is associated with proton translocation across thylakoid membrane, indicating that *Anoectochilus* developed a photo-protective mechanism against photoinhibition (Ruban and Horton 1995). These observations suggest that during HI acclimation *Anoectochilus* modifies its system to dissipate excess excitation energy and to protect the photosynthetic machinery.

References

Amâncio, S., Rebordão, J.P., Chaves, M.M.: Improvement of acclimatization of micropropagated grapevine: photosynthetic competence and carbon allocation. – Plant Cell Tissue Organ Cult. **58**: 31-37, 1999.

Bertamini, M., Nedunchezhian, N.: Decline of photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and soluble protein contents, nitrate reductase and photosynthetic activities, and changes in thylakoid membrane protein pattern in canopy shade grapevine *(Vitis vinifera* L. cv. Moscato giallo) leaves. – Photosynthetica **39**: 529-537, 2001.

Borland, A.M., Técsi, L.I., Leegood, R.C., Walker, R.P.: Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intracellular localization of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. – Planta **205**: 342-351, 1998.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. – Anal. Biochem.**73**: 248-254, 1976.

Chang, D.C.N, Chou, L.C., Lee, K.C.: New cultivation methods for *Anoectochilus formosanus* Hayata. – In: Third International Conference on Mycorrhizas. P. 1. Adelaide Convention Centre, Adelaide 2001.

de las Rivas, J., Abadía, A., Abadía, J.: A new reversed phase-HPLC method resolving all major higher plant photosynthetic pigments. – Plant Physiol. **91**: 190-192, 1989.

Demmig-Adams, B., Adams, W.W., III: The carotenoid zeaxanthin and "high-energy-state quenching" of chlorophyll fluorescence. – Photosynth. Res. **25**: 187-197, 1990.

Demmig-Adams, B., Adams, W.W., III: Photoprotection and other responses of plants to high light stress. – Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 599-626, 1992.

Fryer, M.J., Andrews, J.R., Oxborough, K., Blowers, D.A., Baker, N.R.: Relationship between CO_2 assimilation, photosynthetic electron transport and active O_2 metabolism in leaves of maize in the field during periods of low temperature. – Plant Physiol. **116**: 571-580, 1998.

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. biophys. Acta **990**: 87-92, 1989.

Grout, B.W.W., Aston, M.J.: Transplanting of cauliflower plants regenerated from meristem culture. II. Carbon dioxide fixation and development of photosynthetic ability. – Hort. Res. **17**: 65-71, 1978.

Havaux, M., Niyogi, K.K.: The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. – Proc. nat. Acad. Sci. USA **96**: 8762-8767, 1999.

Hymus, G.J., Ellsworth, D.S., Baker, N.R., Long, S.P.: Does free-air carbon dioxide enrichment affect photochemical energy use by evergreen trees in different seasons? A chlorophyll fluorescence study of mature loblolly pine. – Plant Physiol. **120**: 1183-1191, 1999.

Ito, H., Tanaka, Y., Tsuji, H., Tanaka, A.: Conversion of chlorophyll *b* to chlorophyll *a* in isolated cucumber etioplasts. – Arch. Biochem. Biophys. **306**: 148-151, 1993.

Kornyeyev, D., Holaday, S., Logan, B.: Predicting the extent of photosystem II photoinactivation using chlorophyll *a* fluorescence parameters measured during illumination. – Plant Cell Physiol. **44**: 1064-1070, 2003.

Kozai, T.: Micropropagation under photoautotrophic conditions. – In: Debergh, P.C., Zimmerman, R.H. (ed.): Micropropagation: Technology and Application. Pp. 447-469. Kluwer Academic Publ., Dordrecht – Boston – London 1991.

Krause, G.H.: Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. – Physiol. Plant. **74**: 566-574, 1988.

Pandey, D.M., Kang, K.-H., Yeo, U.-D.: Effects of excessive photon on the photosynthetic pigments and violaxanthin deepoxidase activity in the xanthophylls cycle of spinach leaf. – Plant Sci. **168**: 161-166, 2005.

Pandey, D.M., Wu, R.Z., Hahn, E.-J., Paek, K.-Y.: Drought effect on electrophoretic protein pattern of *Anoectochilus formosanus*. – Sci. Hort. **107**: 205-209, 2006.

Pell, E.J., Eckardt, N.A., Glick, R.E.: Biochemical and molecular basis for impairment of photosynthetic potential. – Photosynth. Res. **39**: 453-462, 1994.

Pfündel, E., Bilger, W.: Regulation and possible function of the violaxanthin cycle. – Photosynth. Res. **42**: 89-109, 1994.

Piel, C., Frak, E., Le Roux, X., Genty, B.: Effect of local irradiance on CO_2 transfer conductance of mesophyll in walnut. – J. exp. Bot. **53**: 2423-2430, 2002.

Powles, S.B.: Photoinhibition of photosynthesis induced by visible light. – Annu. Rev. Plant Physiol. **35**: 15-44, 1984.

Ramalho, J.C., Pons, T.L., Groeneveld, H.W., Azinheira, H.G., Nunes, M.A.: Photosynthetic acclimation to high light conditions in mature leaves of *Coffea arabica* L.: Role of xanthophylls, quenching mechanisms and nitrogen nutrition. – Aust. J. Plant Physiol. **27**: 43-51, 2000.

Rosenqvist, E., van Kooten, O.: Chlorophyll fluorescence: A general description and nomenclature. – In: DeEll, J.R., Toivonen, P.M.A. (ed.): Practical Applications of Chlorophyll Fluorescence in Plant Biology. Pp. 32-77. Kluwer Academic Publ., Dordrecht 2003.

Ruban, A.V., Horton, P.: An investigation of the sustained component of nonphotochemical quenching of chlorophyll fluorescence in isolated chloroplasts and leaves of spinach. – Plant Physiol. **108**: 721-726, 1995.

Slesak, I., Karpinska, B., Surowka, E., Miszalski, Z., Karpinski, S.: Redox changes in the chloroplast and hydrogen peroxide are essential for regulation of C_3-CAM transition and photooxidative stress responses in the facultative CAM plant *Mesembryanthemum crystallinum* L. – Plant Cell Physiol. **44**: 573-581, 2003.

Woitsch, S., Römer, S.: Expression of xanthophyll biosynthetic genes during light-dependent chloroplast differentiation. – Plant Physiol. **132**: 1508-1517, 2003.