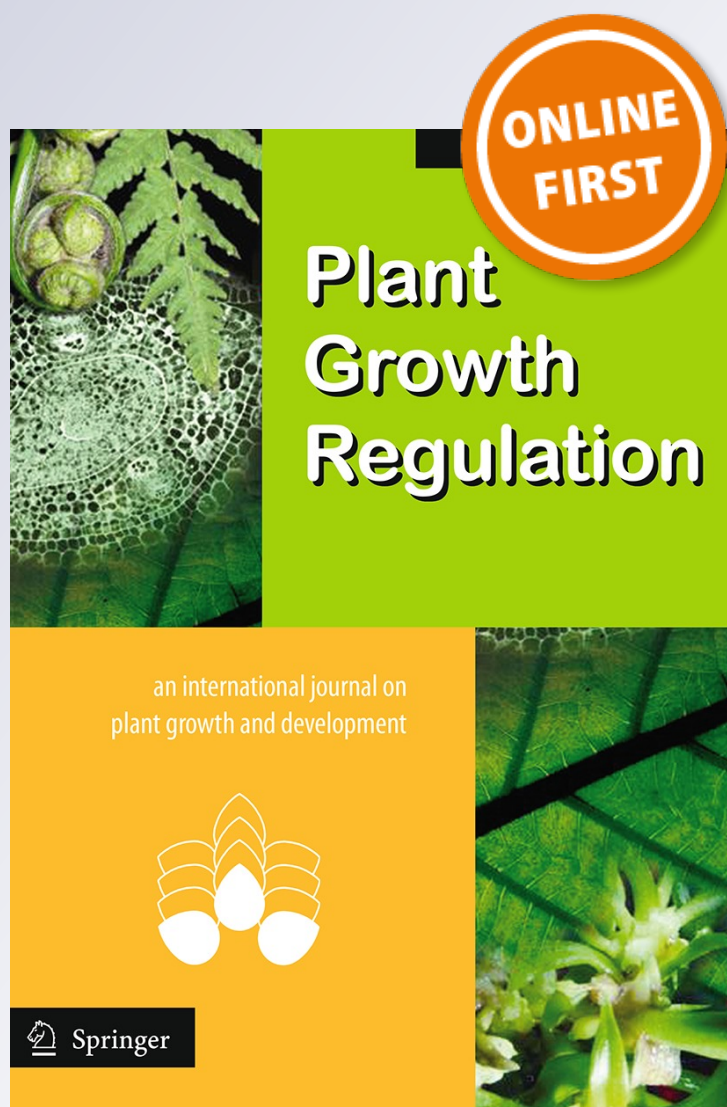# *Effect of photosynthetic photon flux density on growth, photosynthetic competence and antioxidant enzymes activity during* ex vitro *acclimatization of* Dieffenbachia *cultivars*

M. E. El-Mahrouk, Y. H. Dewir, H. N. Murthy, H. Z. Rihan, H. S. Al-Shmgani & M. P. Fuller

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Effect of photosynthetic photon flux density on growth, photosynthetic competence and antioxidant enzymes activity during *ex vitro* acclimatization of *Dieffenbachia* cultivars

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Abstract The effects of 35, 70 and 100 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) were investigated on *ex vitro* acclimatization of micropropagated *Dieffenbachia* plants. Various growth characteristics, photosynthetic parameters and activities of antioxidant enzymes and dehydrins (DHN) were investigated. Fresh and dry plant biomass, plant height and root length were highest under the highest PPFD (100 µmol m^{-2} s^{-1}), but this treatment was responsible for a reduction in the number of leaves. Chlorophyll and carotenoid contents and net photosynthesis were also optimal in plants grown under the highest irradiance. Stomatal resistance, transpiration rate and F_v/F_m values decreased with the incremental light irradiance. Activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase were higher in the plants treated with 70 and 100 µmol m^{-2} s^{-1} PPFD. Accumulation of 55 kDa, 40 and 22 kDa DHN was observed in all light treatments. These results depict that lower PPFD (35 µmol m^{-2} s^{-1}) was suitable for acclimatization of *Dieffenbachia* plants. High PPFD (>70 µmol m^{-2} s^{-1}) induced accumulation of antioxidants and accumulation of DHN in the plants which reveals enhanced stress levels.

Abbreviations

CAT	Catalase
DHN	Dehydrins
F_v/F_m	Maximum quantum yield of photosystem II photochemistry
GPX	Glutathione peroxidase
PPFD	Photosynthetic photon flux density
ROS	Reactive oxygen species
SOD	Superoxide dismutase
RT	Room temperature
FW	Fresh weight
DW	Dry weight

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Introduction

Dieffenbachia is one of the most popular ornamental indoor foliage plants cultivated throughout the world. It has simple and alternate leaves bearing white spots and flecks. There are around thirty recognized species (Mayo et al. 1997) and, in addition, more than a hundred cultivars have been developed through interspecific hybridization and from somaclonal variants produced through tissue culture (Henny and Chen 2004). Even though cultivars of *Dieffenbachia* can be propagated through seeds and vegetative

means, tissue culture methods of propagation are followed by commercial horticulturists due to poor seed production and germination (Henny 1988; Henny and Chen 2004). The tissue culture method of propagation can also enhance the commercialization of new cultivars within 2–3 year compared to 7–10 year through traditional breeding (Shen et al. 2008). However, a major limitation of tissue culture propagation is high mortality of plants during acclimatization (Chandra et al. 2010). This is because of the controlled environmental condition of in vitro culture, which results in the formation of plantlets of abnormal morphology, anatomy and physiology (Pospisilova et al. 1999; Dewir et al. 2006, 2014). Micropropagated plants usually reveal several physiological and anatomical abnormalities during in vitro growth such as low photosynthesis, nonproper functioning of stomata, malfunctioning of housekeeping systems mainly due to high humidity inside the culture vessel (Hazarika 2003; Dewir et al. 2014). After ex vitro transfer, these plantlets might easily be impaired by sudden changed environmental conditions, and so need a period of acclimatization to correct abnormalities (Preece and Sutter 1991; Pospisilova et al. 1999). As a consequence, in vitro raised plants are grown in environmentally controlled growth chambers/green houses and factors such as light, temperature and humidity are gradually enhanced by step-by-step acclimatization of plants (Kirdmanee et al. 1995; Pospisilova et al. 1999; Hazarika 2003).

In order to increase growth and reduce mortality in plantlets at the acclimatization stage, research has been focused on the control of environmental factors which facilitate optimal rate of survival of plantlets (Hazarika 2003; Dewir et al. 2005). Photosynthetic photon flux density (PPFD) is one important factor, which can be controlled at the acclimatization stage, because it can lead to the depression of photosynthetic efficiency (photoinhibition) mainly due to oxidative damage to the photosystem II as in the case of natural environment (Powles 1984). At high PPFD, an increase in photosynthetic carbon fixation can occur, which varies depending on growth and PPFD and may lead to different susceptibilities to photoinhibition (Powles 1984). However, above a certain threshold, carbon fixation becomes saturated and photosynthesis is incapable of using all the energy absorbed by the plants. Under conditions of excess light absorption, the chloroplast lumen becomes acidic in nature, which reduces the electron transport chain, and excitation energy accumulates in the chloroplast which can lead to the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Asada 1999). Such accumulation of ROS may lead to lipid peroxidation and to a reduced accumulation of enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) as a defense system in the plants (Asada 1999). Some

dehydrins (DHN) have been implicated to serve as antioxidants scavenging free radicals (Hara et al. 2003) and may act as protectants of osmoregulation. It is also believed that DHN can accumulate in the acclimatizing plants because it was reported that light induced expression of DHN during seedling de-etiolation in sunflower (Natali et al. 2007). Recently, Dewir et al. (2015) confirmed the accumulation of DHN in acclimatizing African violet plants and that DHN might be playing a role in overcoming lipid peroxidation during ex vitro acclimatization.

Not much work has been carried out on the effect of PPFD during in vitro acclimatization of micropropagated plants and the role of light on antioxidant mechanism. Therefore, in the present study, we carried out ex vitro acclimatization experiments in Dieffenbachia cultivars to study the effect of PPFD on various growth and photosynthetic parameters and the antioxidant enzymes SOD, APX, CAT and DHN in order to clarify their role in overcoming light induced stress during the process of acclimatization.

Materials and methods

Chemicals and reagents

All chemicals were of tissue culture grade, except for enzyme-related analyses, which were of HPLC grade. All reagents and chemicals were obtained from Sigma-Aldrich (Poole, UK), unless otherwise stated.

Plant material and PPFD treatments during acclimatization

Micropropagated plantlets of three Dieffenbachia 'Vesuvius', 'Camille' and 'Green Magic' at the 3–4 leaf stage, were gently removed from in vitro culture medium and the roots were washed under tap water. Plantlets were transplanted into culture pots (300 mL plastic coffee cups with three holes at the bottom) filled with sterilized compost (John Innes No. 1, Westland Ltd., UK) and grown in a cultivation chamber (Sanyo, Japan) for 4 weeks under PPFD of 35, 70 and 100 μmol m^{-2} s^{-1} and a 16-h photoperiod using metal-halide lamps (Sanyo, Japan). The environment in the cultivation chamber was adjusted to 25 $\pm$ 2 °C air temperature and 40–50 % relative humidity (RH). Plants were irrigated twice a week and a compound fertilizer (19: 19: 19; N: P$_2$O$_5$: K$_2$O) at 1 g L^{-1} was applied in the irrigation water once after 3 weeks. Growth parameters such as fresh weight, dry weight, number of leaves/plantlet, shoot and root length, and leaf area were determined after 4 weeks in the growth chamber.

Analysis of photosynthetic parameters

Determination of chlorophyll and carotenoid contents

The amount of chlorophyll-*a*, chlorophyll-*b* and total carotenoids in the fully expanded young leaves were determined by spectrophotometric analysis after 4 weeks in acclimatization. Chlorophyll was extracted from leaf tissue by grinding in a mortar with liquid nitrogen to a fine powder and adding 100 mg to a 2 mL Eppendorf tube. One mL of 80 % acetone was added and the powder was homogenized by inverting for 10 min in ice using a shaker. The absorbance was measured at 470, 649 and 665 nm using a Heliosepiclon spectrophotometer (Unicam, UK). Chlorophyll and carotenoid concentrations were calculated from the spectrophotometric data using the formulae of Lichtenthaler and Welburn (1983). There were three replicates within each treatment.

Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured on the abaxial surface of freshly detached leaf discs. Plants were kept for 30 min in the dark prior to measurement. Modulated fluorescence was measured using a portable chlorophyll fluorometer (PEA, Hansatech Instrument Ltd., version 1.21, Norfolk, UK). Minimal fluorescence (F_0) was measured for 30 min in dark-adapted leaves using light of <0.1 μmol m^{-2} s^{-1} and maximal fluorescence (F_m) was measured after a 1 s saturating pulse (>3500 μmol m^{-2} s^{-1}) in the same leaves. Maximal variable fluorescence ($F_v = F_m - F_0$) and the photochemical efficiency of PSII (F_v/F_m) were calculated for dark-adapted leaves (Dewir et al. 2005). Four plants were randomly selected and measurements were made on the fully expanded young leaves using a standard leaf chamber. There were four single-leaf replications within each treatment.

Stomatal resistance

Stomatal resistance of plant leaves (resistance to loss of water vapour through the stomata; mol H$_2$O m^{-2} s^{-1}) was estimated using an AP4 Porometer (Delta-T Devices Ltd., Cambridge, UK) (Monteith et al. 1988). Four plants were randomly selected and measurements were taken from fully expanded young leaves using a standard leaf chamber. There were four single-leaf replications within each treatment.

Photosynthetic rate and transpiration rate

Photosynthetic rate and transpiration rate were measured with a portable LCi photosynthesis system (ADC BioScientific Ltd., UK). Photosynthetic measurements were carried out under the following conditions: CO$_2$ concentration of the in-flow air was at 350 μmol mol^{-1}, RH at 60 % and leaf temperature was 25 °C. Data was either logged on a PCMCIA-type 1 memory card or was sent directly to a dumb terminal via an RS232 serial link connector. Four plants were randomly selected and measurements were made after 4 weeks on the fully expanded young leaves using a standard leaf chamber. There were four single-leaf replications within each treatment.

Analysis of biochemical parameters

Antioxidant enzymes assay

To determine the activities of antioxidant enzyme, 0.5 g FW of fully expanded young leaves was homogenized under liquid nitrogen with 1.5 mL of extraction buffer using pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 22,000×*g* for 20 min at 4 °C. The supernatant was re-centrifuged at 22,000×*g* for 20 min at 4 °C and was then used for the assays.

Superoxide dismutase assay

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The assay mixture consisted of three reagents (xanthine, xanthine oxidase and cytochrome *c*) dissolved in potassium buffer stored at RT during use and at 4 °C between uses. Xanthine (0.5 mM) was prepared fresh each day and stored at RT during use. SOD anions (O$_2^-$) are generated by a xanthine/xanthine oxidase (XOD) system and detected by a chromagen solution. Xanthine oxidase (5 μL of Sigma X 4500, diluted with 495 μL of potassium phosphate buffer, final concentration, 0.32 U mL^{-1}) was prepared fresh each day and stored on ice during use. Cytochrome *c* (12.5 mg in 0.5 mL) was prepared in phosphate buffer and stored on ice during use and at −20 °C between uses. The assay mixture was prepared from 8.85 mL potassium phosphate buffer, 1 mL xanthine and 50 μL cytochrome. SOD was prepared fresh each day by diluting 10 μL of a Sigma-Aldrich product (S8409, from preparation bovine erythrocytes) with 90 μL buffer (960 U mL^{-1}, final concentration) and stored on ice during use. The assay mix (10 mL) was prepared just before use. This was sufficient for at least 32 wells. Aliquots (50 μL) of sample were added to the wells on a 96-well plate (Fisher Scientific, Loughborough, UK). Some wells were also used as controls either by replacing the sample with SOD or with buffer. Xanthine oxidase solution (100 μL) was added immediately before use to the assay mix, the wells were loaded as appropriate

and readings started within 1 min after this addition. The assay mixture (250 μL) was pipetted into each well and mixed carefully. The plate was immediately transferred to the VersaMax™ plate reader (Molecular Devices, Sunnyvale, CA, USA), and the increase in absorbance was monitored at 550 nm using a kinetic program for at least 5 min. All data are expressed as unit mL^{-1}.

Catalase assay

Catalase (CAT; EC 1.11.1.6) activity was determined according to Clairbone (1985). Aliquots (50 μL) of the leaf extract were added to each well of a 96-well plate (Fisher Scientific), and then 25 μL of 40 μM H_2O_2 was added. The plates were then incubated for 30 min at 37 °C. After incubation, 50 μL of reaction buffer containing 100 μM Amplex Red reagent and 0.4 U mL^{-1} of horseradish peroxidase (Sigma-Aldrich, Poole, UK) was added to each well. The resulting fluorescence was measured using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) with 540 nm excitation and 590 nm emission. The Amplex Red stock solution was prepared at 10 mM in DMSO and aliquots were stored at −20 °C. CAT activity was calculated from a standard curve prepared using purified catalase (0–2000 U mL^{-1}; Sigma-Aldrich).

Glutathione peroxidase (GPX) assay

GPX activity was determined according to Tappel (1978). The assay mixture was prepared by mixing 9.2 mL of buffer (5 mM potassium HEPES, containing 1 mM dipotassium ethylenediaminetetraacetate (EDTA) with 1 mM NADPH, followed by the addition of 100 μL of 100 U mL^{-1} glutathione reductase (GR; Sigma G3664 from *Saccharomyces cerevisiae*), 50 μL of 200 mM glutathione (GSH), and 95 μL of 10 mM potassium cyanide. The assay mixture (290 μL) was added to each well of a 96-well plate, followed by 15 μL of plasma before mixing. The reaction was started by adding 5 μL of 0.042 % (w/w) H_2O_2 solution to each well. The rate of change of absorbance at 340 nm was measured for at least 10 min in a VersaMax™ plate reader. GPX activity was calculated in pmol mL^{-1} min^{-1}.

Immunoblots for DHN proteins detection

DHN proteins were detected according to Rihan et al. (2014) with modifications described by Dewir et al. (2015). Total proteins from the fully expanded young leaves were extracted as described previously by Ni et al. (1996). Samples from −80 °C were thawed on ice, placed in a mortar, and 2 mL of extraction buffer (100 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA, 1 % Triton X-100, 10 % glycerol, pH 7.8) was added to 1 g of tissues and ground with a pestle. One mL of the liquid slurry was transferred to a microfuge tube (1.5 mL) on ice. The supernatant was centrifuged twice at $13,000 \times g$ for 15 and 10 min, respectively, at 4 °C. Total protein content was evaluated using the Pierce BCA assay kit following the manufacturer's instructions. The clear supernatant containing proteins was separated using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Laemmli 1970) and visualized using Coomassie blue staining solution. Unstained gels were used for immunoblot and the proteins were transferred to a PVDF membrane (pore size 0.2 μm) (Millipore, Cat. No. ISE000010) by electro-blotting at 100 V for 35 min. 1× Tris glycine transfer buffer was prepared by dissolving 3.05 g L^{-1} Tris–HCl and 14.4 g L^{-1} glycine in 20 % (v/v) methanol (Towbin et al. 1979). After transferring the proteins onto the PVDF membrane, the membrane was blocked using PBST (phosphate buffer saline + 0.05 Tween 20 %) + 5 % Marvel (a low fat skimmed milk powder) (Iceland Ltd.) for 1 h at RT with mild shaking. After incubation, the membrane was incubated with a primary antibody raised against the DHN K-segment obtained from Dr. Michael Wisniewski's Laboratory, (USDA-ARS, Appalachian Fruit Research Station, USA). Primary antibodies were diluted 1:1000 in a total volume of 20 mL PBST supplemented with 4 % of Marvel semi-skimmed milk and the membrane was incubated at 4 °C overnight with very gentle shaking. The membranes were then washed three times with PBST while agitating to wash off the excess primary antibody, for 5 min each wash, and the membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (PBS diluted 1:20,000) (Abcam) for 1 h at RT with mild shaking to detect primary antibodies attached to the DHN protein. The membranes were washed three times with PBS for 5 min each and then incubated with ECL detection reagent (Luminata crescendo western HRP substrate; Millipore, WBLUR0100) in the dark for 5 min. The image from the membrane was captured using a UVP gel documentation system.

Experimental design and data analysis

All experiments were set up in a completely randomized design. All data were subjected to ANOVA and Duncan's multiple range test (DMRT) using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

Results and discussion

Effect of PPFD on growth parameters

Fresh and dry biomass of plants and root length were highest at 100 μmol m^{-2} s^{-1} PPFD in 'Camille' and

'Green Magic', whereas 70 µmol m^{-2} s^{-1} PPFD was optimum for all these growth parameters with 'Vesuvius' (Table 1). Low PPFD treatment (35 µmol m^{-2} s^{-1}) was superior with respect to the number of leaves per plant and leaf area per plant with 'Camille' and 'Green Magic'. 'Green Magic' gave the highest values for all growth parameters compared to the other two cultivars. In many plant species, the leaves formed in vitro are unable to develop further under *ex vitro* conditions and they are replaced by newly formed leaves (Diettrich et al. 1992). However, if the *ex vitro* transplantation of plantlet is successful, the increase in their growth can be enormous; for example, the *ex vitro* transplanted plants were taller, had higher dry mass, larger leaf area when compared to in vitro plantlets in *Nicotiana tabacum* (Pospisilova et al. 1999).

Effect of PPFD on physiological parameters

Both chlorophyll and carotenoid contents were highest with 100 PPFD in all three cultivars (Table 2). Net photosynthesis values increased with each increment in PPFD in 'Camille' and 'Green Magic', while 'Vesuvius', net photosynthesis values decreased slightly. Thus, *ex vitro* transplantation of plants grown under low irradiance is suggested for successful acclimatization of in vitro raised plants. However, the acclimatization process is a genotype-dependent. An incremental increase in chlorophyll content has been reported in many plant species after *ex vitro* transplantation (Pospisilova et al. 1988; Kozai and Zobayed 2000). Exposure of *Calathea louisae* and

Spathyphyllum floribundum plantlets to high irradiance immediately after transplantation caused photoinhibition and even chlorophyll photo-bleaching (Van Huylenbroeck 1994; Van Huylenbroeck et al. 1995). The increase in photosynthetic activity recorded here showed that plants are able to respond to increases in PPFD but this represents a risk of ROS formation if the energy captured cannot be fixed chemically.

The F_v/F_m was highest with low PPFD (35 µmol m^{-2} s^{-1}) with all three cultivars and F_v/F_m values decreased significantly with increased PPFD (Table 2). The results demonstrate that photoinhibition occurs after transplanting the micropropagated plant, even at low PPFD. Similar decreases in F_v/F_m with increasing PPFD during *ex vitro* acclimatization were also reported with microcuttings of *Rhododendron* during acclimatization (Matysiak 2004). Poorly differentiated chloroplast of in vitro established leaves has been suggested to be the reasons for this result (Lee et al. 1985) and together with water stress, arising from non-functional stomata, which plants have to deal with transplanting (Debergh et al. 2000) results in a low resistance against photoinhibition (Matysiak 2004).

Poorly developed stomata, high stomatal densities and a lack of a thick cuticle are characteristics of micropropagated plants (Pospisilova et al. 1999). Decreased stomatal resistance with increased light intensities was observed in *Dieffenbachia* plantlets (Table 2). In contrast to the current results, low stomatal conductance was reported in *Malaus pumila* plantlets after transfer to *ex vitro* conditions (Diaz-

Table 1 Effect of photosynthetic photon flux density (PPFD) on growth parameters of in vitro raised *Dieffenbachia* cultivars during acclimatization

Cultivar	PPFD	Fresh weight/plant (g)			Dry weight/plant (g)			Plant height (cm)	Root length (cm)	No. of leaves/ plant	Leaf area/ plant (cm^2)
		Shoot	Root	Total	Shoot	Root	Total				
'Vesuvius'	35	2.80 cde[a]	0.41 g	3.22 cde	0.18 d	0.04 e	0.22 de	6.75 cd	3.75 e	4.20 bc	14.89 c
	70	2.81 cde	1.05 b	3.86 cd	0.26 cd	0.07 bc	0.33 cd	9.38 ab	5.38 cd	5.13 bc	25.67 c
	100	2.42 de	1.02 bc	3.43 cde	0.19 cd	0.07 bc	0.26 cde	7.00 cd	4.03 de	3.19 bc	21.79 c
'Camille'	35	2.22 e	0.83 cde	3.05 e	0.17 d	0.06 bcd	0.24 cde	7.88 bc	4.88 cde	4.02 bc	18.69 c
	70	1.88 e	0.50 fg	2.38 e	0.16 d	0.04 d	0.21 e	5.88 d	3.75 e	3.30 c	14.42 c
	100	3.41 cd	0.79 de	4.21 c	0.24 cd	0.05 de	0.29 cde	8.13 bc	5.88 bc	3.36 c	19.70 c
'Green Magic'	35	3.64 c	0.64 ef	4.28 c	0.30 c	0.05 de	0.36 c	9.00 ab	6.75 b	8.75 a	70.04 a
	70	5.66 b	0.91 bcd	6.57 b	0.48 b	0.08 b	0.56 b	10.50 a	9.50 a	5.60 b	44.90 b
	100	7.60 a	1.96 a	9.57 a	0.61 a	0.17 a	0.78 a	9.75 a	6.88 b	5.18 bc	43.56 b
Significance[b]											
Cultivar (C)		***	***	***	***	***	***	***	***	***	***
PPFD		***	***	***	**	***	***	NS	*	NS	NS
C × PPFD		***	***	***	**	***	***	***	***	*	**

[a] Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at at $P \leq 0.05$

[b] *NS* not significant, * significant at $P \leq 0.05$, ** significant at $P \leq 0.01$, *** significant at $P \leq 0.001$

Table 2 Effect of photosynthetic photon flux density (PPFD) on physiological parameters of in vitro raised *Dieffenbachia* cultivars during acclimatization

Cultivar	PPFD	Chlorophyll content (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Net photosynthesis (mol CO_2 m^{-2} s^{-1})	Stomatal resistance (mol H_2O m^{-2} s^{-1})	Transpiration (mol H_2O m^{-2} s^{-1})	F_v/F_m
'Vesuvius'	35	1.39 b[a]	0.17 d	0.07 e	8.25 b	114.26 d	0.80 b
	70	1.29 c	0.23 c	0.21 c	5.38 d	159.41 ab	0.77 c
	100	0.95 e	0.22 c	0.19 c	2.77 e	148.75 c	0.73 d
'Camille'	35	0.93 e	0.22 c	0.16 d	7.8 bc	93.79 e	0.81 ab
	70	1.41 b	0.34 a	0.23 b	6.83 c	161.32 a	0.78 c
	100	1.58 a	0.30 b	0.28 a	2.79 e	157.28 ab	0.74 d
'Green Magic'	35	0.54 g	0.07 f	0.20 c	15.15 a	99.47 e	0.82 a
	70	0.82 f	0.10 e	0.21 c	8.23 b	162.16 a	0.81 ab
	100	1.10 d	0.08 ef	0.26 a	3.13 e	155.03 b	0.64 e
Significance[a]							
Cultivar (C)		***	***	***	***	NS	**
PPFD		***	***	***	***	***	***
C × PPFD		***	***	***	***	***	***

[a] Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$

[b] *NS* not significant, ** significant at $P \leq 0.01$, *** significant at $P \leq 0.001$

Perez et al. 1995). Increments in transpiration rate with an incremental increase in PPFD were observed in all the three *Dieffenbachia* cultivars (Table 2). This might be a result of a decreased development of epi-cuticular waxes as reported in other species, tobacco seedling leaves (Pospisilova et al. 1988) and *Brassica oleracea* (Grout and Aston, 1977; Wardle et al. 1979), *Leucaena leucocephala* (Dhawan and Bhojwani 1987) and *Prunus serotina* (Drew et al. 1992). The combined effects reported on physiological aspects of photosynthesis lead to the conclusion that for Dieffenbachia the low level of PPFD appears to be the safest option for acclimatization as it will lead to a reduced risk of photoinhibition.

Effect of PPFD on antioxidant enzymes

Differential SOD activity was observed with *Dieffenbachia* cultivars exposed to the three levels of PPFD (Fig. 1). SOD accumulation was very low with 'Green Magic', but a fivefold increment in activity was evident with 'Camille'. Among the three light treatments, 100 PPFD was responsible for a stimulated activity of SOD enzyme (Fig. 1a).

The level of CAT activity was similar at all three levels of PPFD treatments (Fig. 1b). 'Green Magic' showed slightly higher levels of CAT activity compared to 'Vesuvius' and 'Camille'. 'Green Magic' showed comparatively lower GPX activity in comparison to 'Vesuvius' and 'Camille' (Fig. 1c). 'Vesuvius' showed a twofold increment of GPX activity when compared to 'Green Magic'. Among the three PPFD treatments, plants exposed to low PPFD (35 μmol m^{-2} s^{-1}) showed comparatively lower

GPX activity, whereas plants exposed to higher PPFD (100 μmol m^{-2} s^{-1}) had a fourfold higher GPX activity in 'Vesuvius' and 'Green Magic'.

It was reported that an inhibition of growth in natural plants is related to physiological and metabolic changes when they are exposed to higher excitation energy, which results in an enhanced accumulation of ROS which can induce oxidative stress (Asada and Takahashi, 1987). The accumulation of ROS in chloroplasts under extreme light stress was reported by Mishra et al. (1995). When ROS are accumulated, a light defensive mechanism will be activated in the plants and various antioxidant enzymes such as SOD, CAT and APX are activated. This was supported in the present study where increased PPFD was responsible for increases of SOD, CAT and GPX levels (Fig. 1). During oxidative stress, SOD destroys superoxide by converting it into hydrogen peroxide, which in turn is detoxified by CAT or GPX reactions (Asada 1999). Similar observations on increased SOD activity during acclimatization phase of plantlets of *Cucurbita pepo* and *Vinca major* have been reported (Logon et al. 1998). Also increased CAT activity has been reported during acclimatization of *Calthea* under increased PPFD (Van Huylenbroeck et al. 2000). It is assumed that the elevated SOD, CAT in combination with GPX in leaves would lower the ROS levels in *Dieffenbachia* cultivars.

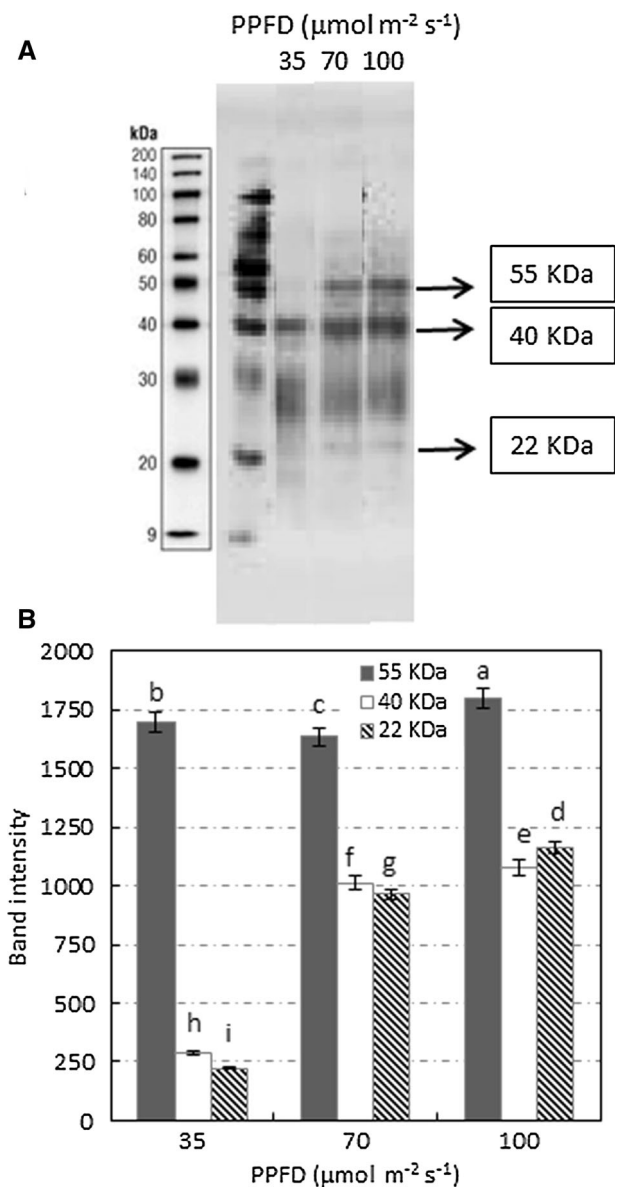### Effect of PPFD on the level of DHN

DHN are a group of proteins that include the late embryogenesis abundant (LEA) proteins which accumulate
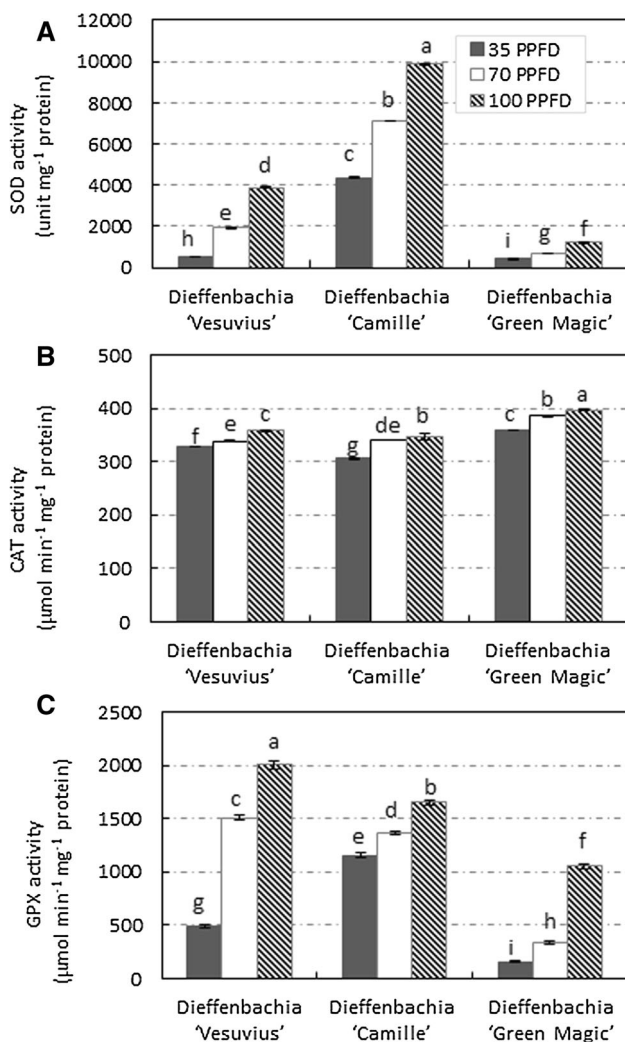
Fig. 1 Activity of antioxidant enzymes in three in vitro raised *Dieffenbachia* cultivars as affected by three PPFDs (35, 70 and 100 μmol m^{-2} s^{-1}) 4 weeks after acclimatization. **a** Superoxide dismutase (SOD); **b** catalase (CAT); **c** glutathione peroxidase (GPX). Values are mean ± standard error (n = 3). *Different letters* above *bars* indicate significant differences between means (Duncan's multiple range test; $P < 0.05$). LSD$_{0.05}$ values 31.72, 3.85 and 37.11 for SOD, CAT and GPX, respectively

Fig. 2 Immunoblot analysis for detection of dehydrins (DHN) in in vitro raised *Dieffenbachia* 'Vesuvius' as affected by three PPFD (35, 70 and 100 μmol m^{-2} s^{-1}) 4 weeks after acclimatization. Values are mean ± standard error (n = 3). *Different letters* above *bars* indicate significant differences between means (Duncan's multiple range test; $P < 0.05$; LSD$_{0.05}$ value = 34.76)

during seed development and are associated with an acquisition of desiccation tolerance in developing seeds (Dure et al. 1981). DHN-like proteins also accumulate in response to other stresses such as cold and salt stress and one such DHN is COR15 which accumulates in response to exposure to low non-freezing temperatures (Close 1997). In recent years, various studies have reported accumulation of DHN in diverse species and tissues in response to abiotic stresses (Kosova et al. 2007). Accumulation of DHN is thought to offer membrane stability during dehydration and confer freezing, drought and salt stress tolerance in *Arabidopsis* and rice (Cheng et al. 2001; Puhakainen et al. 2004). We evaluated the effects of light treatments on

DHN accumulation during acclimatization of *Dieffenbachia* cultivars. The immunoblot analysis of DHN protein fraction extracted from leaves of *Dieffenbachia* cultivars revealed the presence of three major bands with molecular weight of approximately 55, 40 and 22 kDa (Fig. 2). All of these three bands were expressed under exposure to 30, 70 and 100 μmol m^{-2} s^{-1} PPFD in all the three cultivars, however, quantitative differential expression showed that their levels were increased under the highest PPFD (100 μmol m^{-2} s^{-1}). Our results showed the accumulation

of various DHN during light induced stress during *ex vitro* acclimatization and indicate that DHN proteins may be involved in safeguarding cell membranes during light induced stress. Recently, Dewir et al. (2015) reported on accumulation of DHN in relation to a light-induced stress at both low and high PPFD (35 and 100 μmol m^{-2} s^{-1}) during *ex vitro* acclimatization of African violet plants. The results of the present study are in accordance with the work of Hara et al. (2003) and Dewir et al. (2015) who have demonstrated the role of DHN in inhibition of lipid per-oxidation in transgenic tobacco and acclimatizing African violet plants, respectively. We are of opinion that DHN might play a role in overcoming lipid peroxidation in *Dieffenbachia* plants during *ex vitro* acclimatization. However, further investigations are necessary to clarify the role of DHN during *ex vitro* acclimatization of tissue cultured plant species.

Conclusions

In this study, the effect of PPFD (35, 70 and 100 μmol m^{-2} s^{-1} PPFD) on *ex vitro* acclimatization of micropropagated *Dieffenbechia* plants was evaluated. Biomass of plants, plant height, root length, content of chlorophyll and carotenoids, net photosynthesis were optimal in plant irradiated at 100 μmol m^{-2} s^{-1} PPFD. However, this treatment was responsible for high stomatal resistance, enhanced transpiration and it was responsible for accumulation of various antioxidant enzymes as well as DHN proteins. These results clearly depict that enhanced PPFD is responsible for oxidative stress; therefore, low PPFD (35 μmol m^{-2} s^{-1}) is suggested for *ex vitro* acclimatization of micropropagated *Dieffenbachia* plants.

References

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50:601–639

Asada K, Takahashi M (1987) Photoinhibition. In: Kyle DJ, Osmond CB, Arntzen CJ (eds) Production and scavenging of active oxygen in photosynthesis. Elsevier, Amsterdam, pp 227–287

Chandra S, Bandopadhyay R, Kumar V, Chandra R (2010) Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol Lett 32:1199–1205

Cheng ZQ, Targolli J, Su J, He CK, Li F, Wu R (2001) Transgenic approaches for generating rice tolerant to dehydration stress. In:

Khush GS, Brar DS, Hardy B (eds) Rice genetics IV. IRRI Science Publishers, Manila, pp 423–439

Clairbone A (1985) Catalase activity. Handbook of methods for oxygen radical research. CRC Press, Boca Raton, pp 283–284

Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. Physiol Plant 100:291–296

Debergh PC, Topoonyanont N, Van Huylenbroeck JM, Moreira H, Oysert E (2000) Preparation of microplants for *ex vitro* establishment. Acta Hort 530:269–273

Dewir YH, Chakrabarty D, Ali MB, Hahn EJ, Paek KY (2005) Effects of hydroponic solution EC, substrates, PPF and nutrient scheduling on growth and photosynthetic competence during acclimatization of micropropagated *Spathiphyllum* plantlets. Plant Growth Regul 46:41–251

Dewir YH, Chakrabarty D, Ali MB, Hahn EJ, Paek KY (2006) Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. Environ Exp Bot 58:93–99

Dewir YH, Indoliya Y, Chakrabarty D, Paek KY (2014) Biochemical and physiological aspects of hyperhydricity in liquid culture system. In: Paek et al (eds) Production of biomass and bioactive compounds using bioreactor technology. Springer, Berlin, pp 693–703

Dewir YH, El-Mahrouk ME, Al-Shmgani HS, Rihan HZ, Teixeira da Silva JA, Fuller MP (2015) Photosynthetic and biochemical characterization of in vitro-derived African violet (*Saintpaulia ionantha* H. Wendl) plants to *ex vitro* conditions. J Plant Interact 10:101–108

Dhawan V, Bhojwani SS (1987) Hardeing in vitro and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena leucocephala* (Lam.) De Wit. Plant Sci 53:65–72

Diaz-Perez JC, Shackel KA, Sutter EG (1995) Acclimatization and subsequent gas exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. Physiol Plant 95:225–232

Diettrich B, Martinat H, Luckner M (1992) Reduction of water loss during *ex vitro* acclimatization of microporpagated *Digitalis lanata* clone plants. Biochem Physiol Pfanz 188:23–31

Drew AP, Kavanagh KL, Maynard CA (1992) Acclimatizing micropropagated black cherry by comparison with half-sib seedlings. Physiol Plant 86:459–464

Dure L III, Greenway SC, Galau GA (1981) Development biochemistry of cotton seed embryogenesis and germination changing mRNA populations as shown by in vitro and in vivo protein synthesis. J Biochem 20:4162–4168

Giannopolitis CN, Ries SK (1977) Superoxide dismutases I. Occurrence in higher plants. Plant Physiol 54:309–314

Grout BWW, Aston MI (1977) Transplanting of cauliflower plants regenerated from meristem culture I. Water loss and water transfer related changes in leaf wax and to xylem regeneration. Hort Res 17:1–7

Hara M, Terashima S, Fukaya T, Kuboi T (2003) Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin transgenic tobacco. Planta 217:290–298

Hazarika BN (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704–1712

Henny RJ (1988) Ornamental aroids: culture and breeding. Hort Rev 10:1–33

Henny RJ, Chen J (2004) Cultivar development of ornamental foliage plants. Plant Beed Rev 23:245–290

Kirdmanee C, Kitaya Y, Kozai T (1995) Rapid acclimatization of eucalyptus plantlets by controlling photosynthetic photon flux density and relative humidity. Environ Control Biol 33:123–132

Kosova K, Vitamvas P, Prasil IT (2007) The role of deydrins in plant response to cold. Biol Plant 51:601–617

Kozai T, Zobayed SMA (2000) Acclimatization. In: Spier RE et al (eds) Encyclopedia of cell technology. Wiley, New York, pp 1–12

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lee N, Wetzstein HY, Sommer HE (1985) Effects of quantum flux density on photosynthesis and chloroplast ultrastrucutre in tissue-cultured plantlets and seedling of *Liquidambar styraciflua* L. towards improved acclimatization and filed survival. Plant Physiol 78:637–664

Lichtenthaler HK, Welburn AR (1983) Determination of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Logon BA, Demming-Adams B, Adams WW, Grace SC (1998) Antioxidants and xanthophylls cycle dependent energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. acclimated to four growth PPFDs in the field. J Exp Bot 49:1869–1879

Matysiak B (2004) Effect of light intensity on growth and chlorophyll fluorescence of *Rhododendron* microcuttings during acclimatization. Folia Hort 16:107–114

Mayo SJ, Bogner J, Boyce PC (1997) The genera of Araceae. Royal Botanical Gardens, Kew

Mishra NP, Fatma T, Singhal GS (1995) Development of antioxidative defense system of wheat seedlings in response to high light. Physiol Plant 95:77–82

Monteith JL, Cmapbell GS, Potter EA (1988) Theory and performance of dynamic diffusion promoter. Agric For Meterol 44:27–38

Natali L, Giordani T, Lercari B, Maesrini P, Cozza R, Pangaro T, Vernieri P, Martinelli F, Cavallini A (2007) Light induces expression of dehyrin-encoding gene during seedling de-etiolation in sunflower (*Helianthus annus* L.). J Plant Physiol 164:263–273

Ni M, Dehesh K, Tepperman JM, Quail PH (1996) GT-2: in vivo transcriptional activity and definition of novel twin DNA binding domains with reciprocal target sequence selectivity. Plant Cell 8:1041–1059

Pospisilova J, Solarova J, Catsky J, Ondrej M, Opatrny Z (1988) The photosynthetic characteristics during micropropagation of tobacco and potato plants. Photosynthetica 22:205–213

Pospisilova J, Ticha I, Kadlecek P, Haisel D, Plazakova S (1999) Acclimatization of micropropagated plants to *ex vitro* conditions. Biol Plant 42:481–497

Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol 124:15–44

Preece JE, Sutter EG (1991) Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh PC, Zimmerman RH (eds) Micropropagation. Technology and application. Kluwer Academic Publishers, Dordrecht, pp 71–93

Puhakainen T, Hess MW, Makela P, Svensson J, Heino P, Palva ET (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis. Plant Mol Biol 54:743–753

Rihan HZ, Al-Issawi M, Al Shamari M, Woldie W, Kiernan M, Fuller M (2014) The effect of molybdenum on the molecular control of cold tolerance in cauliflower (*Brassica oleracea* var. *botrytis*) artificial seeds. Plant Cell Tiss Org Cult 118:215–228

Shen X, Kane ME, Chen J (2008) Effects of genotype, explant source, and plant growth regulators on indirect shoot organogenesis in *Dieffenbachia* cultivars. In Vitro Cell Dev Biol Plant 44:282–288

Tappel AL (1978) Glutathione peroxidase and hydroperoxides. Methods Enzymol 52:506–513

Towbin H, Staehelint T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76:4350–4354

Van Huylenbroeck JM (1994) Influence of light stress during the acclimatization of in vitro plantlets. In: Struik PC, Vredenberg WJ, Renkema JA, Parlevhet JE (eds) Plant introduction on the threshold of a new century. Kulwer Academic Publishers, Dordrecht, pp 451–453

Van Huylenbroeck JM, Huygens H, Debergh PC (1995) Photoinhibition during acclimatization of microprogagated *Spathiphyllum* "Petie" plantlets. In Vitro Cell Dev Biol Plant 31:160–164

Van Huylenbroeck JM, Piqueras A, Debergh PC (2000) The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. Plant Sci 155:59–66

Wardle K, Quinlan A, Simpkins I (1979) Abscisic acid and the regulation water loss in plantlets of *Brassica oleracea* L. var. *botrytis* regenerated through apical meristem culture. Ann Bot 43:745–752