The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated Calathea plants

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Abstract

The effects of an increased PPFD on photosynthesis, the functioning of the photosynthetic apparatus and the response of the antioxidant enzymatic system were studied during the ex vitro establishment of micropropagated Calathea 'Maui Queen' plantlets. Measured chlorophyll and carotenoids contents in ex vitro formed leaves were almost three times higher compared to the in vitro formed ones. At the end of the acclimatization, an inverse relation between PPFD and the chlorophyll (a + b):carotenoids ratio was observed. During the first days after transplantation Calathea plants are not photosynthetically active, as is illustrated by the photosynthetic light response curves. With the appearance of new leaves, higher photosynthetic capacities were observed and light saturation point increased (days 17 and 25). Also the maximal photosynthetic efficiency enlarged as shown by the increased initial slope of the curves. $F_v/F_m$ decreased directly after transplantation of the micropropagated plantlets, afterwards a recovery was observed, but highest $F_v/F_m$ values were observed in low light (LL) plants. The photochemical quenching coefficient increased gradually during the first two weeks of the acclimatization. In high light (HL) plants, $q_P$ decreased directly after transfer, while this was not observed in LL and medium light (ML). During the acclimatization period to increasing light intensities significant changes in the activity of the antioxidant enzymatic system were observed. A decrease in superoxide dismutase (SOD) activity was measured during the first half of the acclimatization period followed by a recovery in ML and HL plants by day 35. Dehydroascorbate reductase (DHAR) activity decreased during acclimatization. At the end of the experimental period the lowest levels were measured in ML plants. Catalase (CAT) activity increased significantly during the first two weeks after transfer, a clear inverse relationship to PPFD was detected. The relation between the adquisition of full photosynthetic capacity and the activation of the enzymatic antioxidant system in the leaves of calathea plants during ex vitro acclimatization is discussed. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

In vitro culture is an efficient vegetative propagation system for many high-value cultivars. Nevertheless, micropropagation is in some cases restricted due to considerable losses during the ex vitro acclimatization (in greenhouse or field). Therefore improvement in survival and a better knowledge of the physiology of in vitro cultured plants are of considerable interest.

Directly after transfer to ex vitro conditions, micropropagated plants are very susceptible to various stresses, because they have not yet developed adequate patterns of resource allocation and
morphological and physiological features required by the new environment [1]. Low photosynthesis rates [2,3] and the malfunctioning of the water housekeeping system [4] are two of the major constrains of tissue cultured plants. A switch to autotrophy and changes in stomatal functioning and cuticula composition are observed during acclimatization [5–7]. Plantlets are exposed to low photosynthetic photon flux densities (PPFD). Once in the greenhouse, PPFD is much higher and plants become very susceptible to light stress. Excessive radiation during acclimatization can even lead to chronic photoinhibition [8,9]. Besides photoinhibition, plants also suffer due to the difference between in vitro and ex vitro relative humidity [10]. Both phenomena, photoinhibition and water stress, can generate toxic O₂ species [11–13]. As protection against the latter, plant cells have developed several antioxidant and enzymatic scavenging systems as superoxide dismutase (SOD), catalase (CAT), peroxidase, dehydroascorbate reductase (DHAR) and glutathione reductase (GR) [14]. Accordingly, there are many examples relating SOD and enzymes of the H₂O₂ scavenging pathway to environmental stresses in plants [15]. However, limited information on the possible role of these protective enzymatic systems during the acclimatization process and their relation with photoinhibition and malfunctioning in the transpiration system of micropropagated plants is available. Recently, it was demonstrated that micropropagated plants develop antioxidant mechanisms during acclimatization [16].

In the present study we investigated the effects of an increased PPFD during the ex vitro establishment of micropropagated Calathea ‘Maui Queen’ on photosynthesis, the functioning of the photosynthetic apparatus and the enzymatic activities in the scavenging systems of activated oxygen.

2. Materials and methods

2.1. Plant material and light treatments

Calathea louisae Gagnep. ‘Maui Queen’ plantlets were regularly micropropagated according to Dunston and Sutter [17], at three months intervals, on agar-solidified medium containing 3% sucrose and 2% glucose. Cultures were maintained at 22 ± 1°C under cool-white fluorescent lamps (PPFD at plant level 30 μmol m⁻² s⁻¹ and a 16 h photoperiod). For acclimatization, rooted shoots were transplanted to a peat substrate and placed in a growth room (21 ± 1°C, RH 80–90%, CO₂ concentration 350 ppm, day–night regime 16 h light and 8 h darkness). Three different PPFDs were applied (40 μmol m⁻² s⁻¹, low light, LL; 120 μmol m⁻² s⁻¹, medium light, ML; 360 μmol m⁻² s⁻¹, high light, HL).

Leaf samples were taken at transplanting (day 0) and after 4, 7, 11, 16, 26, 42 days and stored in liquid nitrogen. Photosynthesis and chlorophyll a fluorescence parameters were measured at day 0, 3, 6, 10, 17, 25 and 40.

2.2. Photosynthesis measurements

Net CO₂ gas exchange rates were measured as described in Van Huylenbroeck et al. [8], using a portable ADC-LCA3 photosynthesis measuring system and a temperature and light controlled modified Parkinson Leaf Chamber (PP-Systems, Hertfordshire, United Kingdom). Dark respiration rates (μmol CO₂ m⁻² s⁻¹) as well as CO₂ uptake rates (μmol CO₂ m⁻² s⁻¹) at a PPFD of 40, 120 and 360 μmol m⁻² s⁻¹ were measured. During measurements leaf temperature was controlled at 20°C and CO₂ concentration was 350 ml l⁻¹.

2.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence on the adaxial side of the last fully developed leaf was measured with a pulse-amplitude-modulation fluorometer (Model PAM-2000, Walz, Effeltrich, Germany) as described earlier in detail [8]. Minimal fluorescence (F₀) was determined after 15 min dark adaptation, while maximal fluorescence (Fₘₐₓ) was measured after a saturation pulse. The fluorescence ratio Fᵥ/Fₘₓ, with Fᵥ = Fₘₓ − F₀ being the variable fluorescence, was then calculated. Photochemical quenching (qᵥ) at three different light intensities was measured, using the saturation pulse method. Hereto plants were subsequently illuminated during 4 min with continuous actinic light of 40, 120 or 360 μmol m⁻² s⁻¹. At each light intensity, saturation pulses were given at 20 s intervals during the last 2 min of illumination. Afterwards a 4 s long far red illumination was given to determine F₀. Calculations of qᵥ were made according to van Kooten and Snel [18].
2.4. Pigment determination

Pigments were extracted in darkness using the methanol–chloroform (1:3 v/v) procedure of Quail et al. [19]. Chlorophyll a and b as well as total carotenoids in the chloroform layer were calculated according to Wellburn [20].

2.5. Enzyme extraction and assays

For enzyme determination approximately 1 g fresh weight of plant tissue was homogenized in 3 ml pre-cooled extraction buffer consisting of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 2.5 mM PMSF, 8% (w/v) insoluble PVP and 0.2% Triton X-100. The homogenate was filtered through cheesecloth (Miracloth) and centrifuged three times during 5 min at 10 000 × g at 4°C. The supernatant was used for enzyme assay. The protein concentration was determined according to Lowry et al. [21] using bovine serum albumin as a standard. All enzymes were assayed spectrophotometrically at 25°C.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) was measured according to Foyer and Halliwell [22]. The reaction mixture contained 50 mM phosphate buffer (pH 6.8) and 2.5 mM glutathione. The reaction was started with dehydroascorbic acid (0.5 mM). The absorption coefficient for ascorbate at 265 nm is 14 mM cm⁻¹. Control rates in the absence of enzyme extract were subtracted.

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined according to Foyer and Halliwell [23]. The assay contained 50 mM Tris buffer (pH 7.8), 1.0 mM EDTA and 0.12 mM NADPH (absorption coefficient at 340 nm: 6.22 mM cm⁻¹). The reaction was started with 0.8 mM GSSG. Control rates obtained in the absence of GSSG and of enzyme extract were subtracted.

Ascorbate peroxidase (1.11.1.7) activity was determined and extracted as described by Amako et al. [24]. The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (absorption coefficient 2.8 mM cm⁻¹). Guaiacol peroxidase activity was measured according to Chance and Maehly [25] using a reaction mixture with 50 mM phosphate buffer (pH 7) and 5 mM guaiacol (absorption coefficient at 436 nm: 25.5 mM cm⁻¹). The reaction was started with 20 mM hydrogen peroxide.

Catalase (CAT) (EC 1.11.1.6) activity was measured as described by Aebi [26]. The decomposition of H₂O₂ was monitored by the decrease in absorbance at 240 nm. For the assay a 50 mM phosphate buffer (pH 7.8) and 10 mM H₂O₂ were used.

Superoxide dismutase (SOD) (EC 1.15.11) activity was determined by the cytochrome c method using xanthine–xanthine oxidase as the source of superoxide radicals, and a unit of activity was defined according to McCord and Fridovich [27].

2.6. Statistical analysis

A two factor analysis of variance (ANOVA) was conducted. When significant differences occur, mean separation was performed using the LSD (P = 0.5) method. Data are presented ± standard errors.

3. Results

3.1. Pigments

No relation between total chlorophyll or carotenoid content and PPFD was observed in
leaves of *Calathea* (Fig. 1(A) and (B)). Pigment concentration increased significantly about three weeks after transplantation when new leaves appeared. Measured chlorophyll and carotenoids contents in those new leaves were almost three times higher compared to the in vitro formed ones (Fig. 1(A) and (B)). Chlorophyll a to b ratio increased from 1.95 to 2.2 ± 0.1 (Fig. 1(C)). The maximal ratio was reached faster in ML and HL plants. The chlorophyll (a + b)/carotenoids ratio of LL plants increased slightly during acclimatization (Fig. 1(D)). On contrary, a decrease in this ratio was found during the first days for ML and HL plants. At the end of the acclimatization, an inverse relation between PPFD and the chlorophyll (a + b)/carotenoids ratio was observed (Fig. 1(D)).

3.2. Photosynthesis and fluorescence measurements

During the first days after transplantation *Calathea* plants are practically not photosynthetically active as is illustrated by the photosynthetic light response curves (Fig. 2). From day 6 on, positive values were measured, although saturation was already reached at very low PPFD. With the appearance of new leaves, higher photosynthetic capacities were observed and light saturation point increased (day 17 and 25). Also the maximal photosynthetic efficiency enlarged as was shown by the increased initial slope of the curves. At the end of the experimental period (day 40) an inhibition of photosynthesis was observed for ML and HL plants, while net photosynthesis for LL plants continued to increase.

Photosynthetic efficiency is also evaluated by chlorophyll fluorescence measurements (Figs. 3 and 4). \( F_v/F_m \) decreased directly after transplantation of the micropropagated plantlets. This decrease was significantly stronger with increasing PPFD, and was mainly due to a reduction in \( F_m \) (Fig. 3). Afterwards a recovery was observed, but highest \( F_v/F_m \) values were observed in LL plants.

![Fig. 2. Evolution of light response curves as measured at different moments during the acclimatization of micropropagated *Calathea* ‘Maui Queen’ plantlets grown at a PPFD of 40 (●), 120 (■) or 360 (○) \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Values are means ± S.E. (n = 6) (if not visible, S.E. smaller than data label).](image)

![Fig. 3. Fluorescence parameters \( F_v/F_m \) (A), \( F_o \) (B) and \( F_m \) (C) during acclimatization of micropropagated *Calathea* ‘Maui Queen’ at a PPFD of 40 (●), 120 (■) or 360 (○) \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Values are means ± SE (n = 6) (if not visible, S.E. smaller than data label).](image)
3.3. Enzymatic activities

For SOD a decrease in activity was observed during the first half of the acclimatization period followed by a recovery in ML and HL plants by day 35, while the activity in LL plants remained unchanged (Fig. 5(A)). Ascorbate peroxidase activity increased slightly in time, the highest values were measured for ML and HL plants at day 14 and 35 (Fig. 5(B)). DHAR activity decreased during acclimatization. At the end of the experimental period lowest levels were measured in ML plants (Fig. 5(C)). CAT activity increased significantly during the first two weeks after transfer (Fig. 5(D)). A clear inverse relationship to PPFD (highest activities measured in LL plants) was observed. As observed for CAT, also GR activity doubled during the acclimatization of micropropagated Calathea plantlets (Fig. 5(E)). However, for GR no correlation with PPFD was found. Guaiacol peroxidase activities remained unchanged for LL plants, while a significant increase for ML and HL plants was observed at the end of the acclimatization (Fig. 5(F)).

4. Discussion

Directly after transplantation, CO$_2$ fixation by in vitro leaves of Calathea was very low (Fig. 2). Downregulation of photosynthesis in vitro is frequently observed during micropropagation, due to depletion of CO$_2$ in the culture vessels [28] and...
feedback inhibition of the Calvin cycle by exogenous sugar supply resulting in deactivation of Rubisco [29]. Also Grout and Aston [2] measured a negative CO₂ balance in cauliflower and this was the case until two weeks after plants had been transferred to soil. During this period plants are very susceptible to additional stresses. Also in this study, an increase in light intensities had strong photoinhibitory effects, as was reflected by a fast decrease of the variable over the maximal fluorescence ratio (Fig. 3). A similar process was observed during acclimatization of micropropagated Spathiphyllum plants [7]. The low q_p values found during the same period (Fig. 4) indicates that a high proportion of the primary quinone electron acceptor of PSII is in a reduced state and that a low percentage of the captured light energy is directed through the electron transport chain [9].

Starting from day 10, a switch from heterotrophic to autotrophic metabolism is observed and functional photosynthetic apparatus are developed, as is reflected by the increased light saturation point and photosynthetic efficiency (Fig. 2). In addition to this metabolic switch, the photoinhibitory effects of increased light intensities diminished, as evidenced by the recovery of the F_v/F_m ratio (Fig. 3). In this period new ex vitro formed leaves and functional roots were fully developed and plants acquired complete autotrophic metabolism. Previous results have already demonstrated that the in vitro formed leaves of Calathea never become fully autotrophic [7]. However, prolonged exposure of the ex vitro formed leaves to ML and HL conditions had still an inhibitory effect on photosynthetic activity (lower F_v/F_m decreasing net photosynthesis at day 40). These results are in agreement with observations in micropropagated Liquidambars [30].

With the formation of new leaves, a strong increase in chlorophyll and carotenoids amounts are seen (Fig. 1). Donelly and Vidaver [31] also found higher pigment contents in ex vitro formed leaves of Rubus. The decrease of chlorophyll content at day 40 for ML and HL plants, proved the photodamaging effect of a prolonged exposure to higher light intensities. The chlorophyll a to b ratio increased during acclimatization, as was reported by Donelly and Vidaver [31] for Rubus. On the other hand, a reverse relationship between light intensity and chlorophyll (a + b)/carotenoids ratio was observed (Fig. 1). This relative increase in carotenoids observed in ML and HL plants reflects a flexible functional response of the photosynthetic apparatus to the different light environments, since the photoprotective role of carotenoids against photooxidative damage is well documented [32,33].

The changes observed in the antioxidant enzymatic activities during acclimatization of Calathea indicate a progressive activation of this enzymatic system against oxidative stress. During the acclimatization of micropropagated plants, two situations of environmental stress able to generate activated oxygen species may occur: photoinhibition [8] and moderated water stress [34,35]. Some of the changes observed in our experiment for SOD, CAT, GR and ascorbate peroxidase activities during the acclimatization period resemble those found in plants exposed to mild water stress [36,35]. Enhancements in the activity of activated oxygen-scavenging enzymes during water or other environmental stress situations probably occurs in the chloroplasts, because in photosynthetic tissues, ascorbic acid-dependent antioxidants as ascorbate peroxidase and GR are predominantly localised in the chloroplasts, which are the major sites of H₂O₂ production in leaves [37]. The activity of antioxidant enzymes may also be important for the scavenging of toxic O₂ species in other cell compartments. There is increasing evidence indicating the importance of the cytosolic isoforms of antioxidative enzymes in plants grown under various environmental stress conditions [38,39]. In response to changes in light intensity SOD, CAT, ascorbate peroxidase, GR and guaiacol peroxidase activities showed clear changes for each acclimatization date. The increase of SOD, ascorbate peroxidase and GR activities under high light conditions reveal a protection against photooxidative stress linked to photoinhibition, as it has been previously reported by Logan et al. [39] in Cucurbita and Vinca plants exposed to increasing light intensities. The rise in many of the antioxidant activities, that are located in different cellular compartments, suggest that formation of activated oxygen species can augment with increasing light intensity not only in chloroplasts, but also in mitochondria and peroxisomes. Since approximately 90% of total leaf ascorbate peroxidase activity is localised in the chloroplast [40], this activity can represent the best estimation of chloroplast-based detoxification of reactive oxygen.
species via the Mehler-peroxidase pathway as sink for reducing equivalents and/or a mechanism to increment the transthylakoid membrane pH gradient [41,42]. In the case of GR, by the end of the acclimatization period this activity was inhibited at higher light intensities. GR utilises NADPH to re-reduce oxidized glutathione. Since this compound is the reductive substrate for the enzyme DHAR that reduces the oxidation product of ascorbate, chloroplastic GR could be considered as part of the Mehler-peroxidase pathway. Our results showed that the acclimatory pattern in relation to light intensity for DHAR is correlated to some extend to GR although it differed from those of SOD and ascorbate peroxidase by the end of the acclimatization period. There could be several possibilities to explain this. In first place, chloroplasts have two pathways to reduce MDA (monodehydroascorbate): direct photoreduction as well as an enzymatic reduction via MDA reductase using GR [43]. In addition GR activity may be influenced by the need for reduced glutathione for cellular processes not associated with the Mehler-peroxidase pathway as for example sulphur metabolism and maintenance of protein redox balance [44]. From all the enzymes studied in this work only CAT activity exhibited an inverse relation to light intensity. This result is not surprising since it is well documented that catalase synthesis and degradation is light sensitive [45] and high light intensities accelerate this process in *Calathea* leaves. The observed pattern of activity for CAT in relation to light intensity strengthens the significance of the acclimatory changes observed for the predominantly chloroplastic ascorbate peroxidase. It is possible that the pattern observed for CAT is related to its role in photorespiration and antioxidant enzymatic protective system during acclimatization of *Calathea* plants at the end of the acclimatization, when new leaves are fully developed.

Our work suggests that tissue-cultured plants develop a functional photosynthetic apparatus and antioxidant enzymatic protective system during acclimatization. A good climatic control during the critical days of the acclimatization process will lead to better results.

References

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