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Photosynthetic ability of in vitro grown coconut (*Cocos nucifera* L.) plantlets derived from zygotic embryos

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Abstract

Photosynthetic parameters have been investigated using complementary approaches throughout the in vitro development of coconut zygotic embryos into plantlets. Patterns of chlorophyll fluorescence were comparable in in vitro grown coconut plantlets ($\theta_P^{MAX} = 0.72$ and $\theta_P = 0.45$) and in autotrophic adult palms ($\theta_P^{MAX} = 0.76$ and $\theta_P = 0.50$). Chlorophyll content was lower in in vitro-cultured plantlets (0.92 mg/g fresh weight (FW)) than in autotrophic plants (2.43 mg/g FW). The photosynthetic rate (1.14 μmol CO_2/m^2 per s) of autotrophic palms was half that of in vitro grown plantlets, while transpiration rates were similar in both. Changes in the PEPC:RubisCO ratio during the development of in vitro grown plantlets (from 89.17 to 0.04 μmol CO_2/h per mg total soluble protein (TSP)) reflected a transition from a heterotrophic towards a RubisCO-mediated mode of CO_2 fixation. The RubisCO enzyme capacity (2.83 μmol CO_2/h per mg TSP) and content (172.8 mg/g TSP) measured in in vitro-cultured plantlets were lower than those measured in autotrophic palms (6.60 μmol CO_2/h per mg TSP and 217.6 mg/g TSP respectively). Transmission electronic microscopy (TEM) observations showed a complete ultrastructural organisation of chloroplasts in plantlets at the end of the in vitro culture process (6 weeks under light). All the studied parameters/

Abbreviations: Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b*; FW, Fresh weight; PAR, Photosynthesis active radiations; PEPC, Phosphoenolpyruvate carboxylase; PVP, Polyvinyl pyrrolidone; RH, Relative humidity; RubisCO, Ribulose 1,5-bisphosphate carboxylase oxygenase; T°, Temperature; TCA cycle, Tricarboxylic acid or Krebs cycle; TEM, Transmission electronic microscopy; TSP, Total soluble proteins.

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have shown that plantlets at the end of the in vitro culture process exhibit photosynthetic characteristics (θ_P^{MAX}, θ_P, PEPC:RubisCO ratio and transpiration rates) similar to those of acclimatized plants. These results suggest an early establishment of a photosynthetic metabolism during the in vitro development of coconut plantlets. Nevertheless, RubisCO content and capacity together with chlorophyll content were found to remain lower in in vitro grown plantlets, which might explain the lower photosynthetic rates recorded, as compared to the autotrophic coconut palm.

Keywords: Chlorophylls; Embryo culture; Fluorescence; PEPC; Photosynthesis; RubisCO

1. Introduction

Due to the high weight, large size and lack of dormancy of coconut (*Cocos nucifera* L.) seeds, in vitro culture of zygotic embryos provides a useful alternative for collecting and exchanging germplasm [1]. Sampling methodology and in vitro culture protocols for coconut zygotic embryos have been well documented [2–6]. Nevertheless, when compared to seedlings, coconut in vitro grown plantlets show a slower development in the nursery after acclimatization [7]. Thus, the intrinsic quality of in vitro grown coconut plantlets needs to be improved. Furthermore, zygotic embryo culture provides a model system which could be applied to improve conditions used for in vitro development of somatic embryos [8,9].

During the transfer to ex vitro conditions, the physiological status of in vitro grown plantlets is an important factor determining success rates [10,11]. In order to optimize this very critical phase, apart from the control of water stress [12], investigations on the photosynthetic ability of plantlets during the in vitro process are necessary.

The coconut palm, like many in vitro grown C_3 plantlets, can assimilate inorganic carbon via two independent carboxylation pathways. One occurs in the chloroplasts through the action of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO, EC:4.1.1.39). The activity of this enzyme is closely linked to the development of chloroplasts and reflects the integrity of the photosynthetic apparatus. The other pathway takes place in the cytosol through the phosphoenolpyruvate carboxylase pathway (PEPC, EC:4.1.1.31) [13–16,5,17]. PEPC is an ubiquitous, highly regulated enzyme in plants [18]. The C_3-PEPC feeds carbon into the Tricarboxylic acid (TCA) cycle to provide precursors for various biosynthetic processes, including amino acid biosynthesis [19]. Thus the ratio of PEPC:RubisCO activities could be a reliable indicator of the relative conditions of non-photosynthetic (heterotrophic) and photosynthetic (autotrophic) pathways of CO_2 fixation [20]. Nevertheless, the carboxylase activities, as measured in vitro on protein extracts, can only give an estimation of the optimal capacity for CO_2 fixation in the plant material. These enzymatic studies need to be complemented with investigations on in planta photosynthetic parameters. The measurement of fluorescence emission from the chlorophylls of photosynthetic systems provides a non-invasive approach to study the photochemical events of photosynthesis and provides accurate information on the activity of the photosynthetic apparatus through the efficiency of photosystem II (PSII) [21,22]. This technique has already been successfully applied to in vitro grown plant material in order to assess in vitro photosynthesis [23–25,16,17].

The photosynthetic ability of plantlets needs to be confirmed by CO_2-exchange measurements in planta. This approach has been developed for in vitro culture of various plant species such as strawberry, potato, tobacco and rose [26–28,25,11].

To date, in coconut, the photosynthetic characteristics of in vitro grown plantlets have not been intensively studied. The purpose of the work reported here was to investigate the photosynthetic status of in vitro grown coconut plantlets obtained by zygotic embryo culture, combining various complementary approaches applied both in vitro and in planta.

2. Material and methods

2.1. Plant material

Zygotic embryos of the autogamous Malaysian Yellow Dwarf (MYD) coconut (*Cocos nucifera* L.) ecotype were collected in Indonesia, Mexico and Côte d'Ivoire. The MYD ecotype was chosen because it exhibits autogamous reproduction, thus heterogeneity between zygotic embryos can be controlled and it gives satisfactory results in embryo culture [1,2]. An acclimatized, 6 year-old autotrophic coconut palm, cultivated in a computer monitored tropical glasshouse, was used as a standard.

2.2. In vitro culture parameters

The tissue culture medium was composed of Murashige and Skoog [29] micro- and macroelements modified by Rabéchault and Martin [30], Morel and Wetmore [31] vitamins, 60 g/l sucrose and 2 g/l activated charcoal, according to the protocole described by Assy-Bah et al. [7]. The pH was ajusted to 5.0 before adding charcoal, then the medium was autoclaved (110°C, 103 Kpa, 20 min). Zygotic embryos were collected and cultured as previously described [1], except that liquid medium was used throughout the in vitro culture protocol [5].

Excised coconut embryos were grown in Pyrex tubes (*h*, 18 cm, Φ, 2.4 cm) in the dark (Temperature (T°), 27 ± 1°C). They were transferred every 2 months onto 20 ml fresh liquid medium. As soon as the first leaf and a complete root system were fully developed, plantlets were transferred on to 100 ml liquid medium in one litre culture bottles under light (45 ± 5 μmol/m^2 per s). Plantlets were acclimatized when they were 6 months old and displayed 2–3 unfolded green leaves.

2.3. Procedure for the biochemical analysis

2.3.1. Fresh plant material

Mature embryos, etiolated leaves (harvested from shootlets just before transfer to the light), greening leaves and mature leaves from plantlets ready for acclimatization were sampled for analysis. Green leaves were collected 1–2 h after the beginning of the light period. All biochemical analyses were performed in triplicate, on at least three different samples.

2.4. Chlorophyll fluorescence

Chlorophyll fluorescence from intact leaves of in vitro grown plantlets was measured with a modulated fluorescence measurement system (Hansatech, England). The leaf was placed between the parallel jaws of the clip connected to the fluorimeter and illuminated with a modulated light source which produced a minimal photosynthetic effect. The initial level (*F*o) of chlorophyll fluorescence corresponding to all PSII reaction centres in the open configuration (i.e. with Q_A oxidized) was determined by shining 8 μmol/m^2 per s of 583 nm light. A short pulse (1 s; 8000 μmol/m^2 per s) was used to induce the maximal fluorescence level (*F*m) corresponding to all PSII reaction centers in the closed state (i.e. with Q_A reduced). Afterwards, the leaf was exposed to continuous actinic light (40 μmol/m^2 per s) and illuminated with pulses (1 s every 10 s) of 8000 μmol/m^2 per s in order to determine the maximum fluorescence level (*F*'m) in the light. Fluorescence increased rapidly to a peak and slowly declined to a steady-state level (*F*s) [22]. In the dark-adapted leaf, an index for the maximal quantum yield of photochemistry through PSII (θ_P^{MAX}) was calculated as $(Fm - F0)/Fm$ [32]. The actual quantum yield of PSII photochemistry in light-adapted leaves (θ_P) was calculated as $(F'm - Fs)/F'm$ [33].

2.5. Measurement of CO_2 exchanges and transpiration rates

Net in vivo photosynthesis was estimated through the measurement of CO_2 exchange by intact leaves. We used a LI-COR LI-6400 portable photosynthesis system (LI-COR, Nebraska, USA) coupled with a 6 cm^2 standard leaf chamber equipped with a CO_2/H_2O sensor head and a 6400-02 type LED light source. For

measurements on in vitro growing leaves, the atmosphere in the leaf chamber was programmed according to the atmosphere detected in the tissue culture bottle itself ($[CO_2]$, 517.9 ± 72.0 ppm, photosynthesis active radiations (PAR), 50 μmol/m^2 per s, relative humidity (RH), $61.9 \pm 8.4\%$). The airflow rate was set at 140 ml/min. Results were expressed as μmol CO_2/m^2 per s for net photosynthesis rates and as mmol H_2O/m^2 per s for transpiration rates.

During the operation, the in vitro grown plantlet was temporarily removed from the bottle and one unfolded leaf was clamped in the leaf chamber. Roots were maintained in the liquid medium during this time. Values for photosynthesis and transpiration were recorded after a 2–3 min stabilisation period. At least two measurements were performed on two different leaves for each plantlet.

2.6. Estimation of chlorophyll concentrations

Leaf pigments were extracted at 4°C in 80% (v/v final concentration) acetone over a period of 12 h in the dark. Concentrations of chl *a* and *b* were calculated according to Lichtenthaler and Wellburn [34].

2.7. Enzyme extraction and measurement of PEPC and RubisCO capacities

Samples averaging 100 mg fresh weight (FW) of mature embryos or fresh leaves were collected for analysis. PEPC and RubisCO were extracted according to Nato and Mathieu [35]. In order to protect proteins against phenolic compounds, the extraction buffer was supplemented with insoluble PVP (660 mg/g FW) according to Rival et al. [5]. PEPC and RubisCO capacities were assayed on crude extracts following the incorporation of ^{14}C-labelled sodium bicarbonate into acid stable products as previously described [36]. For both enzymes, crude extracts were incubated for 30 min (30°C). Total soluble protein (TSP) contents were estimated on crude enzyme extracts, according to Bradford [37].

2.8. Quantification of RubisCO

Quantification of RubisCO in protein crude extracts was performed by rocket immunoelectrophoresis according to the method of Laurell [38], modified by Lavergne et al. [15]. Polyclonal antibodies raised against the spinach RubisCO holoenzyme were kindly provided by Dr Piquemal (CNRS Toulouse, France). Purified RubisCO from tobacco leaves was used as a calibration protein.

2.9. Numeration and ultrastructure of chloroplasts

Leaf samples (4×1 mm), collected from in vitro grown plantlets and from adult acclimatized autotrophic palm were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h and rinsed three times for 30 min in the same cacodylate buffer.

Leaf samples were post-fixed in 1% (v/v) buffered Osmium tetroxide for 2 h at 4°C and rinsed three times with distilled water. After progressive dehydration using an ethanol series (10–100%), samples were embedded in Spurr's resin [39].

For ultrastructural observations, sections (80–90 nm) were made with an ultramicrotome (Reichert Ultracut Leica) and were contrasted by immersion in 2% uranyl acetate (30 min in the dark, room temperature) followed by a 10 min immersion in a lead citrate solution (1.33 g lead citrate + 1.76 g sodium nitrate in 50 ml alkaline aqueous solution, pH 12). Observations and microphotographs were carried out using a transmission electronic microscope (JEOL 100C).

For the counting of chloroplasts, 2 μm sections were obtained as previously described and colored with periodic-shiff acid and Naphthol Blue Black [40] before observation through a light microscope (Leica Orthoplan) at $G = 100$.

2.10. Statistical test

The significance of mean differences was studied using ANOVA analysis of variance and the Newman [41] and Keuls test [42] (95%), using Statistica® StatSoft software.

Table 1
Changes in the maximal quantum yield (θ_P^{MAX}) of PSII photochemistry (in dark-adapted leaves) and the actual quantum yield (θ_P) of PSII photochemistry (in light-adapted leaves) in coconut leaves sampled at various stages of in vitro development.

Culture stage	θ_P^{MAX} $(Fm-F0)/Fm$	θ_P $(F'm-Fs)/F'm$
Dark grown plantlet	0.29 ± 0.02a	ND
1 week PAR	0.58 ± 0.02a	0.33 ± 0.03a
2 weeks PAR	0.71 ± 0.05c	0.41 ± 0.09b
4 weeks PAR	0.72 ± 0.04c	0.45 ± 0.03b
Autotrophic plant	0.76 ± 0.01c	0.50 ± 0.03b
ANOVA		
F	138.67	6.94
P	0.0000	0.0033

Reported values are the means of three independent measurements ± S.D.
Results of one way analysis of variance (ANOVA) are given: Snedecor's variable (F) and Type I Error (P).
Values followed by the same letter (a, b, c, d, e) were not significantly different as determined by the Newman and Keuls' test.

3. Results

3.1. Chlorophyll fluorescence

Chlorophyll fluorescence parameters (θ_P and θ_P^{MAX}) were measured in dark-grown leaves and during greening under PAR (Table 1). θ_P^{MAX} was very low in dark-grown plantlets. This parameter increased during the greening of leaves as early as 2 weeks after cultivation under PAR. Values for θ_P and θ_P^{MAX} were not significantly different in in vitro grown plantlets (after 4 weeks under PAR) and in the acclimatized coconut palm.

Table 2
Net photosynthesis estimated through CO_2 exchanges and transpiration rates in leaves from in vitro grown coconut plantlets

Culture stage	Photosynthetic rate μmol CO_2/m^2 per s	Transpiration rate mmol H_2O/m^2 per s
Dark grown plantlet	-0.500^a	0.040^a
1 week PAR	0.271^{ab}	0.340^a
2 weeks PAR	0.940^b	1.135^b
4 weeks PAR	1.144b	1.140^b
Autotrophic plant (reference)	2.430^c	1.137^b
ANOVA		
F	11.40	9.458
P	0.0006	0.0027

Values followed by the same letter (a, b, c, d, e) were not significantly different as determined by the Newman and Keuls' test.

Table 3
Changes in chlorophyll content and Chl a/Chl b ratio during the in vitro development of coconut zygotic embryos into plantlets

Culture stage	Total chlorophyll $(a+b)$ (mg/g FW)	Chl a/Chl b ratio
Mature embryo	0.005 ± 0.000^a	0.625 ± 0.739^a
Dark grown plantlet	0.071 ± 0.080^a	0.678 ± 0.080^a
1 week PAR	0.134 ± 0.106^a	3.695 ± 0.313^b
2 weeks PAR	0.456 ± 0.035^a	3.249 ± 0.568^b
4 weeks PAR	0.329 ± 0.190^a	2.600 ± 0.705^b
6 weeks PAR*	0.921 ± 0.228^b	3.661 ± 0.227^b
Autotrophic plant	1.451 ± 0.277^b	3.144 ± 0.092^b
ANOVA		
F	30.81	35.97
P	0.0000	0.0000

Reported values are the means of three independent measurements ± S.D..
* Ready for acclimatization.
Values followed by the same letter (a, b, c, d, e) were not significantly different as determined by the Newman and Keuls' test.

3.2. Net photosynthesis and transpiration

Net photosynthesis rates were measured through CO_2 exchange in leaves from in vitro grown plantlets (Table 2). During the greening of leaves, the net CO_2 exchange increased. The photosynthesis rate in in vitro-cultured plantlets was then half of that measured in the autotrophic coconut palm.

3.3. Transpiration

The transpiration rate in in vitro dark-grown plantlets was 0.04 mmol H_2O/m^2 per s (Table 2). During the greening of leaves, transpiration increased up to a value which was not significantly different from the rate measured in an autotrophic palm.

Table 4
Changes in total soluble protein contents and RubisCO and PEPC specific capacities during the in vitro development of coconut zygotic embryos

Culture stage	TSP content (mg/g FW)	PEPC capacity (μmol CO_2/h per mg TSP)	RubisCO capacity (μmol CO_2/h per mg TSP)	PEPC:RubisCO ratio
Mature embryo	14.40 ± 1.71^{b}	17.83 ± 0.51^{a}	0.20 ± 0.00^{a}	89.17 ± 2.56^{a}
Dark grown plantlet	0.94 ± 0.00^{a}	40.30 ± 11.31^{a}	0.95 ± 0.21^{ab}	42.14 ± 2.49^{a}
1 week PAR	3.07 ± 0.49^{a}	4.17 ± 2.55^{b}	2.12 ± 1.02^{bc}	2.31 ± 1.44^{b}
2 weeks PAR	5.18 ± 0.25^{a}	3.14 ± 0.79^{b}	5.41 ± 1.19^{d}	0.61 ± 0.24^{b}
4 weeks PAR	5.13 ± 1.09^{a}	1.38 ± 1.00^{b}	3.83 ± 0.41^{c}	0.36 ± 0.26^{b}
6 weeks PAR*	20.56 ± 3.38^{c}	0.08 ± 0.03^{b}	2.83 ± 0.34^{bc}	0.03 ± 0.01^{b}
Autotrophic plant	14.26 ± 5.09^{b}	0.33 ± 0.36^{b}	6.60 ± 1.56^{d}	0.04 ± 0.04^{b}
ANOVA				
F	49.47	19.40	23.22	121.34
P	0.0000	0.0000	0.0000	0.0000

Changes in total soluble protein contents, RubisCO and PEPC specific capacities during the in vitro development of coconut zygotic embryos.
Reported values are the means of three independent measurements ± S.D.
* Ready for acclimatization.
Values followed by the same letter (a, b, c, d, e) were not significantly different as determined by the Newman and Keuls' test.

3.4. Chlorophyll content

During the development in the dark of zygotic embryos, very low chl (*a* and *b*) levels were found, as expected (Table 3). After exposure to light, the total chlorophyll content increased up to 0.921 mg/g FW in green plantlets just before acclimatization (i.e. 6 weeks under PAR). At this stage, the chlorophyll content was not significantly different in the autotrophic palm as compared with the in vitro grown plantlets.

Chl *a*/Chl *b* ratios were low at the beginning of the culture process when plantlets were cultivated in the dark. From one week under PAR onwards, this ratio was found not to be significantly different in in vitro grown plantlets, as compared with that measured in an autotrophic palm.

3.5. RubisCO and PEPC capacities and PEPC:RubisCO ratio

Carboxylase (PEPC and RubisCO) capacities were measured in mature zygotic embryos, etiolated leaves and greening leaves (Table 4).

In the mature embryo, the TSP content was high, while in greening leaves lower TSP contents were measured. A significant increase was noted after 6 weeks under light.

During the development of embryos into plantlets, the PEPC capacity decreased concomitantly with an increase in RubisCO capacity. The PEPC capacity was high during the early stages of in vitro culture. At the same time, the RubisCO capacity remained at a very low level.

During the in vitro culture process, the PEPC capacity of leaves drastically decreased. In contrast, the RubisCO capacity increased throughout the in vitro culture period. Consequently, the PEPC:RubisCO ratio dropped from 89.17 in the mature embryo down to 0.03 in ready-to-acclimatize plantlets (6 weeks under PAR), a ratio similar to that found in the autotrophic coconut palm.

3.6. Quantification of RubisCO

RubisCO was quantified in mature embryos, dark grown plantlets and in greening leaves. An example of the patterns of rocket immunoelectrophoresis obtained for RubisCO from in vitro zygotic embryo-derived plantlets is given in Fig. 1. RubisCO content, which was measured throughout in vitro greening of leaves, increased from 0

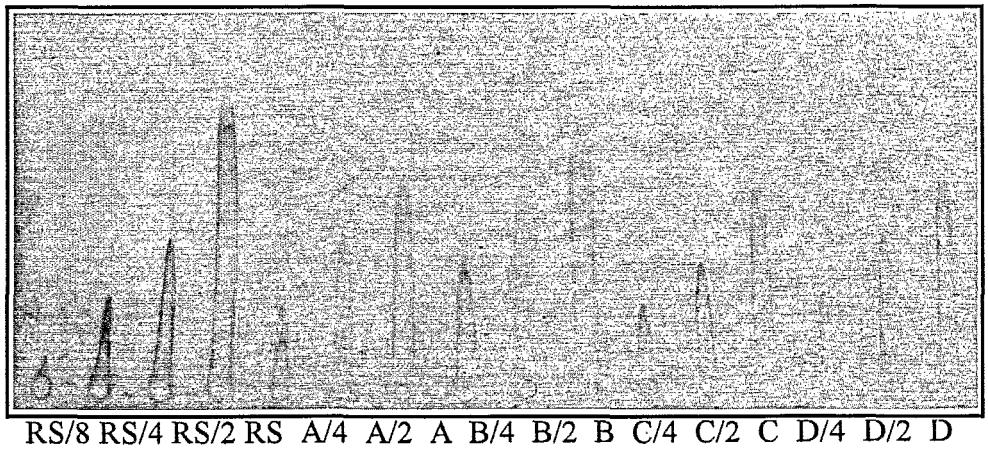

Fig. 1. Rocket immunoelectrophoresis patterns obtained for RubisCO protein extracted from in vitro grown coconut plantlets. TSP content was adjusted to 150, 180, 200 ng in sample RS, A and B respectively and 120 ng in samples C and D. Samples A, B, and C were extracted from 3 different in vitro coconut plantlets at the same stage (4 weeks under PAR illumination). Sample D was from an adult acclimatized coconut palm. RS was commercial RubisCO protein purified from tobacco leaves, used as standard. X/2 to X/8 are progressive dilutions of the same extract.

mg/g TSP in dark-grown leaves to 172.8 mg/g TSP in leaves after 4 weeks under PAR (Fig. 2). The RubisCO content was found to be 217.6 mg/g TSP in the autotrophic coconut palm.

3.7. Counting and ultrastrustural visualisation of chloroplasts

Chloroplast counts and transmission electronic microscopy (TEM) ultrastructural analysis was performed on in vitro grown plantlets before acclimatization. The number of mature chloroplasts did not significantly differ between in vitro grown leaves (5.6 ± 1.3 chloroplasts per cell) and leaves from adult autotrophic palm (5.7 ± 0.6). Ten cells from three leaf sections were observed for each sample. Completely differentiated grana and numerous thylakoïds could be observed by TEM in chloroplasts from leaves of in vitro grown plantlets (Fig. 3) as well as in leaves of autotrophic coconut palms.

4. Discussion

Our results demonstrate the establishment of photosynthetic metabolism during the in vitro development of coconut plantlets. Several notable similarities have been observed between in vitro grown coconut plantlets and the adult autotrophic coconut palm.

At the end of the in vitro culture process, mature chloroplasts containing numerous grana and well-developed thylakoids can be observed in green leaves. Similar results have been reported for in vitro-cultured plantlets of *Gardenia jasminoides* [43]. In contrast, the presence of flattened chloroplasts with irregularly arranged internal membranes has been described as a characteristic of in vitro grown plantlets of *Liquidambar styraciflua* [44,45] and rose [46]. For these species, the lack of internal chloroplast membrane suggests that the photosynthetic capacity of in vitro grown plantlets may be lower than that of seedling-derived plants. In coconut, our observations of a functional chloroplast ultrastructure are consistent with several photosynthetic parameters investigated in the present study.

Our results suggests that there is a high level of PSII activity in the vitroplant. Our data are consistent with the θ_P^{MAX} values obtained for oil palm using both in vitro grown (0.74) and acclimatized plantlets (0.79) [17] or measured in several species cultivated in vitro, such as tobacco (0.82) or potato (0.73) [25] or field-grown species such as, for example, *Solanum tuberculosum* (0.83) or *Trifolium repens* (0.82) [47,48].

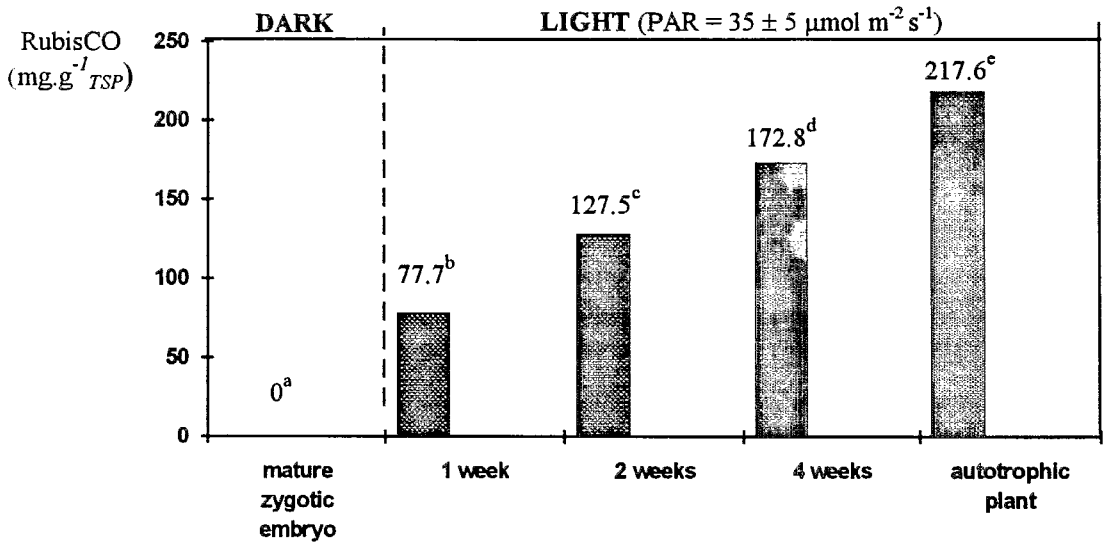

Fig. 2. Estimation by immunoelectrophoresis of RubisCO content in the leaves of in vitro grown coconut plantlets.

θ_P is a reliable index of quantum yield of PSII photochemistry in illuminated leaves [49] and reflects, for coconut, a fully functional linear electron transport chain in in vitro grown coconut plantlets. This was not the case for in vitro grown oil palm plantlets, which showed a θ_P value (0.23) only half that of acclimatized plants (0.54) [17].

The chlorophyll fluorescence emission and chloroplast ultrastructure results suggest that in vitro grown coconut plantlets develop an efficient photosynthetic apparatus, comparable to the one observed in an autotrophic palm [21,22] at an early stage.

CO_2 exchange measurements have revealed an increase in the net photosynthesis rate of in vitro-cultured plantlets. Both photosynthesis and chlorophyll fluorescence were found to increase concomitantly during the in vitro culture process, suggesting an increase in CO_2 assimilation in the plantlets. The existence of a correlation between θ_P and CO_2 fixation measurements under non-photorespiratory conditions has been reported by many authors [49,21,22]. Nevertheless, the photosynthesis rate measured in in vitro grown plantlets remained half as much as that of the autotrophic palm. Generally, higher photosynthesis rates are recorded in seed-derived plants as compared with in vitro grown material [28]. It must be noted that the value obtained for the adult autotrophic coconut palm, which was cultivated in a tropical glasshouse (2.43 μmol CO_2/m^2 per s), was much lower than the one measured with the same ecotype cultivated in natural tropical conditions and therefore under a markedly different light and temperature regime (11 ± 5 μmol CO_2/m^2 per s) [50].

Many authors reported that unacclimatized leaves of in vitro cultured plantlets showed permanent stomatal opening or poor control of water loss [51,12]. In vitro-cultured *Asparagus* plantlets were found to show higher transpiration rates than acclimatized plants [52]. Our observation that transpiration rates are similar in in vitro-cultured plantlets and in the autotrophic palm suggests that stomatal opening is correctly regulated in in vitro coconut plantlets.

The low photosynthetic rate measured in in vitro grown coconut plantlets was accompanied by a lower chlorophyll content as compared with the autotrophic palm. The same observation was made for oil palm plantlets cultivated in vitro [17]. The chlorophyll content measured for in vitro grown coconut plantlets was of the same order as that determined for example, in tobacco in vitro grown plantlets (1.09 mg/g FW) [25]. The Chl *a*/*b* ratio measured in in vitro grown coconut plantlets

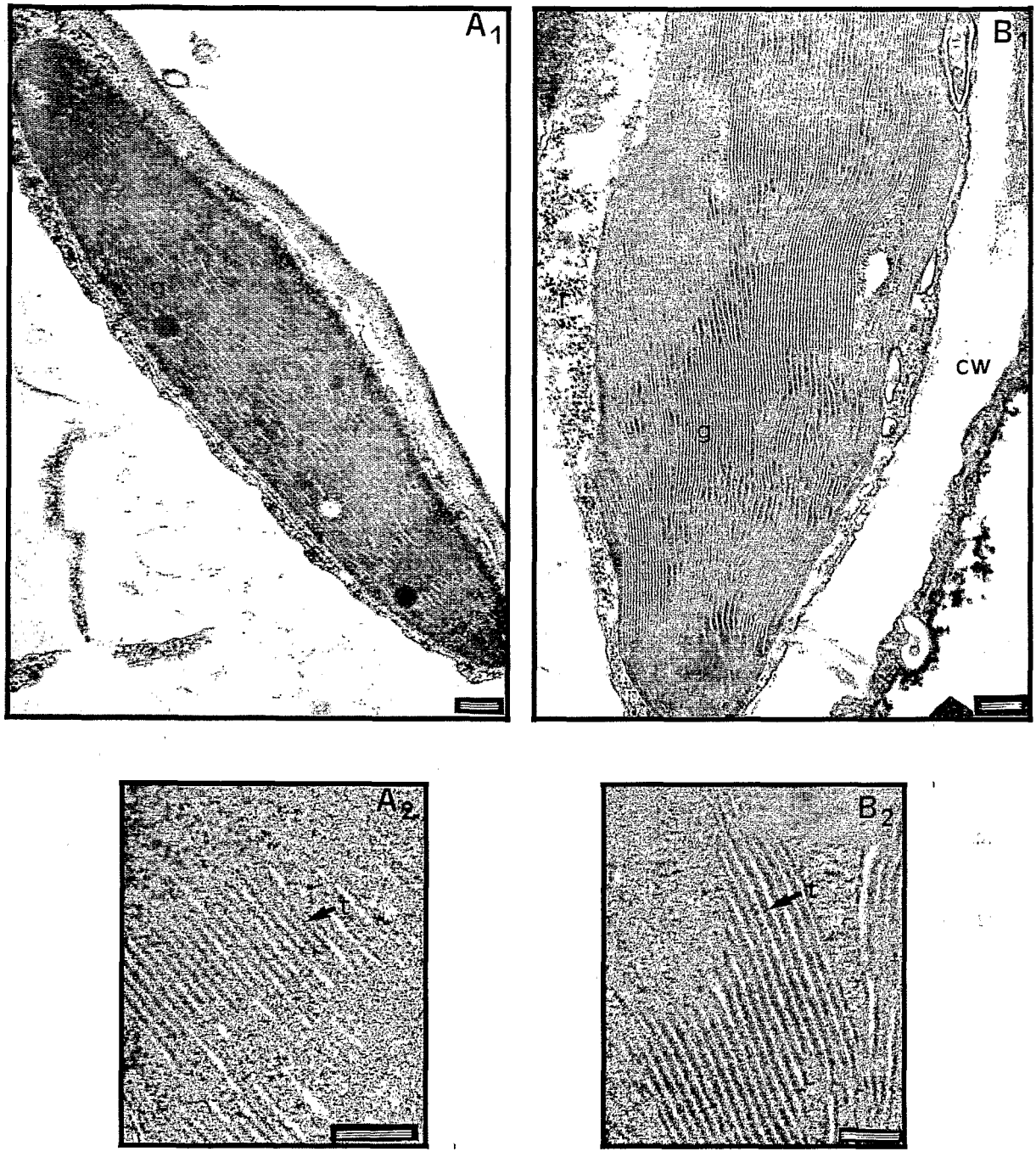

Fig. 3. Comparison of chloroplast ultrastructure in leaves from coconut in vitro grown plantlets (a) and in the autotrophic adult palm (b). A1, B1: chloroplasts ultrastructure, Scale bar, 200 nm. A2, B2: details of chloroplast ultrastructure, Scale bar, 100 nm. cw: Cell wall, g: Grana, r: Ribosomes, t: Thylakoids.

was comparable to that measured in vitroplants from other species [43,45]. This ratio was found not to be significantly different in the adult autotrophic palm than in the in vitro grown plantlet from any culturing stage. Lower Chl *a/b* ratios have been demonstrated in tissue-cultured plantlets than in seedlings for *Liquidambar styracifolia* [45], but this was apparently not the case for coconut. The very low Chl *a/b* ratio measured in dark-grown leaves is presumed to be an artifact, as the spectrocolorimetric method used for this study [34] was at its lower limit of detection in the latter case. Thus, the extremely low (< 0.1 mg/g FW) chlorophyll content measured was of the same order of magnitude as background fluctuations.

At the early stages of in vitro culture (i.e. 1 week under PAR) the PEPC:RubisCO ratio was very high, due to a high PEPC capacity. High PEPC capacities (6.6 μmol CO_2/h per mg TSP) were also measured in shoot-forming cotyledons of *Pinus radiata* [20] and in young somatic embryos of oil palm (5.2 μmol CO_2/h per mg TSP) [17]. Thus, during the early stages of culture, CO_2 fixation is maintained through the anaplerotic PEPC-pathway. C_3-PEPC plays a pivotal role in the integration and coordination of C- and N-metabolism. The occurrence of a transient C_4-type behaviour (preferential CO_2 fixation through PEPC) when C_3 plants or isolated cells are cultivated in vitro has been described in several species [14,53,54]. The PEPC:RubisCO ratio decreased in in vitro grown plantlets down to 0.03, a value similar to the one measured in autotrophic coconut palm. Similar patterns were observed in in vitro grown plantlets of oil palm [5], in which a depletion of the PEPC:RubisCO ratio (down to 0.06) was noted during the in vitro development of somatic embryos.

During in vitro growth and development, coconut plantlets showed a transition from a heterotrophic to an autotrophic (RubisCO-mediated) mode of carbon fixation. Indeed, a marked decrease in PEPC, concomitant with substantial increase in RubisCO capacity, was observed. This phenomenon has been reported to occur in in vitro cultures of various C_3 species, such as strawberry or oil palm [16,17]. In our case, the RubisCO capacity and content were lower than in the adult autotrophic coconut palm and it could explain the low rates of CO_2 assimilation found in in vitro grown plantlets. The high level of sucrose present in the culture medium (60 g/l) could affect the RubisCO capacity. Indeed, exogenous carbohydrates have been reported to induce a depletion in RubisCO efficiency [11,14,55,56] and photosynthetic rate [43]. A reduction of the sucrose level in culture medium at the end of the in vitro process could therefore allow an increase in photosynthesis, probably via an increase in RubisCO efficiency. Photoautotrophic micropropagation, implemented under high PAR flux and a CO_2-enriched atmosphere, has been described by many authors ([57], for a review). In the case of coconut, the need for transferability of the in vitro culture process to producing countries requires the use of simpler protocols. In addition, in the case of coconut palm it would probably be difficult to decrease the level of sucrose in culture media because of the unusual seed physiology of this species. Coconut seednuts are characterized by considerable levels of carbohydrates reserves [58,59], upon which the zygotic embryo, and subsequently the plantlet, may depend for 18 months in average [60].

5. Conclusion

The work described here provides a multiparameter approach to the study of photosynthesis in in vitro cultivated coconut plantlets, from the zygotic embryo to the fully developed plantlet.

In vitro-cultured coconut plantlets displayed an early initiation of a photosynthetic metabolism. Concomitant changes in several parameters (θ_P^{MAX}, θ_P, CO_2 fixation, PEPC:RubisCO ratio and transpiration rates) were measured. However, a lower rate of net photosynthesis was recorded in in vitro grown plantlets as compared with the acclimatized palm. This could be explained by a lower RubisCO content and activity, together with a lower chlorophyll content compared to the acclimatized palm.

Given the fact that high amounts of NO_3 are used in the culture medium [29] and that significant nitrate and nitrite reductase activities have previously been measured in in vitro cultured plant cells [61,62], it can be assumed that part of the photosynthetic electron flow is used for the reduction of nitrates [63–65] which accumulate within cells during the active root growth occurring at the culture stage in question. This could explain the lower photosynthetic activity measured in in vitro grown plantlets as compared with an autotrophic palm, at a similar level of electron flow (θ_P).

This work now needs to be complemented by the monitoring of the parameters studied here during the subsequent stage of acclimatization of plantlets. A comparative analysis of leaf anatomy in in vitro grown plantlets and in acclimatized coconut palm should also be performed, as plantlets cultivated in vitro have been reported to produce leaves with few palissade cells and large intracellular spaces, hence affecting the availability and the assimilation of CO_2 [66].

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