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EXOGENOUS SUCROSE CAN DECREASE *IN VITRO* PHOTOSYNTHESIS BUT IMPROVE FIELD SURVIVAL AND GROWTH OF COCONUT (*COCOS NUCIFERA* L.) *IN VITRO* PLANTLETS

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Summary

Coconut (*Cocos nucifera* L.) plantlets grown *in vitro* often grow slowly when transferred to the field possibly, due to a limited photosynthetic capacity of *in vitro*-cultured plantlets, apparently caused by the sucrose added to growth medium causing negative feedback for photosynthesis. In this paper, we tested the hypothesis that high exogenous sucrose will decrease ribulose 1,5-bisphosphate carboxylase (Rubisco) activity and photosynthesis resulting in limited *ex vitro* growth. Plantlets grown with high exogenous sucrose ($90\,g\,l^{-1}$) had reduced photosynthetic activity that resulted in a poor photosynthetic response to high levels of light and CO_2. These plantlets also had low amounts of Rubisco protein, low Rubisco activity, and reduced growth despite showing high survival when transferred to the field. Decreasing the medium's sucrose concentration from 90 to $22.5\,g\,l^{-1}$ or $0\,g\,l^{-1}$ resulted in increased photosynthetic response to light and CO_2 along with increased Rubisco and phosphoenolpyruvate carboxylase (PEPC) activities and proteins. However, plantlets grown *in vitro* without exogenous sucrose died when transferred *ex vitro*, whereas those grown with intermediate exogenous sucrose showed intermediate photosynthetic response, high survival, fast growth, and *ex vitro* photosynthesis. Thus, exogenous sucrose at moderate concentration decreased photosynthesis but increased survival, suggesting that both *in vitro* photosynthesis and exogenous sucrose reserves contribute to field establishment and growth of coconut plantlets cultured *in vitro*.

Key words: A/C; curve; chlorophyll fluorescence; field survival; *in vitro*; pigmen

Introduction

Coconut palms cultured *in vitro* often show slow growth and establishment when transferred *ex vitro* (Ashburner, 1994; Ashburner et al., 1995). It has been suggested that poor survival and slow field establishment of tissue-cultured plantlets once transferred to the field is due to their poor photosynthetic efficiency (Santamaría et al., 1999; Carvalho et al., 2001). However, other authors believe that field establishment and *ex vitro* growth of *in vitro* plantlets is not necessarily related to *in vitro* photosynthesis (Yué et al., 1993). The high exogenous sucrose content in the medium has been shown to considerably diminish the photosynthetic ability of plantlets cultured *in vitro* (de la Viña et al., 1999; Arigita et al., 2002). Exogenous sugar in the culture medium could suppress photosynthetic gene expression, reduce chlorophyll content, reduce Calvin cycle enzymes, as well as reduce Rubisco activity and Rubisco concentration, leading to low photosynthetic rates (Sheen, 1990; Krapp et al., 1991; Schäfer et al., 1992; Hdider and Desjardins, 1995; Koch, 1996; Van Huylenbroeck and Debergh, 1996; Santamaría et al., 1999; Sinha et al., 2002). Yet, exogenous sugars, in some cases, improve photosynthesis *in vitro* and improve the acclimatization success (Capellades et al., 1991; Kovtun and Daie, 1995; Van Huylenbroeck and Debergh, 1996; Tichá et al., 1998; de la Viña et al., 1999).

Preliminary work in our laboratory showed that 6-mo.-old coconut seedlings had two-fold higher photosynthetic rates than coconut plantlets grown *in vitro* with $45\,g\,l^{-1}$ of exogenous sucrose. The aim of this work was to test the hypothesis that high sucrose concentration in the medium leads to feedback inhibition of photosynthesis, that in turn, could be related to the slow growth during acclimatization of plantlets to field conditions.

Materials and Methods

Plant material and culture conditions. Zygotic embryos extracted from the seeds (nuts, 12 post-anthesis) of 15-yr-old coconut palms (Malayan Green Dwarf) grown in the field were cultured *in vitro* in solid medium (Peche et al., 2002) with $45\,g\,l^{-1}$ of sucrose for 6–8 wk at a room temperature of 27 ± 2°C in the dark. Germinated embryos were then cultured in 16/8 h light/dark photoperiod, 27 ± 2°C, at a photosynthetic photon flux density (PPFD) of $50\,\mu mol\,m^{-2}\,s^{-1}$, 80% relative humidity, in liquid medium Y3 (Eeuwens, 1976; modified by Rillo and Paloma, 1992) for an additional 8 wk. Plantlets were then cultivated in four different concentrations of exogenous sucrose: 0.0, 22.5, 45.0, or $90.0\,g\,l^{-1}$ for an additional 32 wk. Subcultures

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were made every 8 wk to fresh Y3 liquid medium containing the treatment exogenous sucrose concentration. The plantlets were placed in a standard growth-room with a 16/8 h light/dark photoperiod, light intensity of 50 μmol PPFD $m^{-2}s^{-1}$ provided by cool white fluorescence lamps (Phillips 39 W), and temperature of 27 ± 2°C, using 500 ml glass containers with 50 ml of liquid medium. Coconut seedlings used as controls were germinated and grown under field (full sun) conditions in pots with sterile sand: soil substrate (1:1 proportions) under 40–95% relative humidity, 0–2000 μmol PPFD $m^{-2}s^{-1}$, and temperatures of 20–40°C.

Photosynthetic rate. A portable LI-6400 (LI-COR Inc., Lincoln, Nebraska, USA) was used to measure net assimilation of CO_2 (A_{CO2}) in response to increasing levels of light and in response to increasing internal leaf CO_2 (C_i). Light intensity ranged from 0 to 1500 μmol PPFD $m^{-2}s^{-1}$ at a constant CO_2 concentration of 370 μmol mol^{-1}. C_i concentrations ranged from 0 to 1000 μmol mol^{-1} at a constant light intensity of 600 μmol PPFD $m^{-2}s^{-1}$. The functional photosynthetic parameters were calculated by fitting rectangular hyperbola to the measured points using the software Photosynthesis Assistant (1.1.2 for Windows by Parsons and Ogston, Dundee Scientific, UK). Parameters examined included apparent quantum efficiency (AQE), carboxylation efficiency (CE), CO_2 compensation point (Γ_{CO2}), light compensation point (Γ_L), respiration (Resp), dark respiration (Rd), maximum photosynthesis (Amax), and saturation point (Psat).

Pigment contents. Chlorophylls and carotenoids were determined in the youngest fully expanded leaf (YFEL) using 100 mg fresh weight (FW) extracted with 10 ml of 80% cold acetone at 4°C, as described by Wellburn (1994).

Chlorophyll fluorescence. Efficiency Analyzer (PEA) Hansatech (Norfolk, UK) was used to measure leaf chlorophyll fluorescence parameters in a 24 h cycle, after darkening-adaptation of the leaf for 20 min, in the abaxial side of intact YFEL: Fo (minimum fluorescence), Fv (Fm − Fo; variable fluorescence), Fm (maximum fluorescence), and Fv/Fm (variable/maximum ratio fluorescence; efficiency of electron transport of photosystem II; PSII).

Enzyme extractions. One g of frozen samples from YFEL was ground with a mortar and pestle with liquid nitrogen and with 20% (w/v) of polyvinylpolypyrrolidone (PVPP; Nato and Mathieu, 1978). Two ml (w/v) of Tris extraction buffer were added to the resulting powder. The buffer contained 100 m*M* Tris-HCl pH 8.0, 1 m*M* phenylmethylsulfonylfluoride (PMSF), 5 m*M* dithiothreitol, and 10 m*M* $MgCl_2$ at 4°C. The homogenate was centrifuged at 12000 × *g* for 20 min. The supernatant was then used for protein analysis, total enzyme activity (described below), and content assays.

Total soluble protein. Total soluble proteins (TSP) were determined in crude extracts in YFEL as described by Bradford (1976). Albumin bovine serum was used for standard curves.

Rubisco and PEPC assays. Crude enzymatic extracts were prepared from samples of fresh leaves, according to Nato and Mathieu (1978). Ribulose 1,5-bisphosphate (Rubisco, EC 4.1.1.39) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) total capacities were assayed following the incorporation of $NaH^{14}CO_3$ into acid-stable compound from YFEL as described in Nato and Mathieu (1978). Ten μg of the enzyme extract (described above) with Tris assay buffer were pre-incubated by adding $NaH^{14}CO_3$ (Accesolab, NEC-086H, 2.5 μCi per assay vial, 55 mCi $mmol^{-1}$) at 30°C for 2.5 min. The assay for Rubisco was started by adding 2 m*M* 1,5-ribulose bisphosphate (sodium salt) and incubated at 30°C for 10 min. The assay was stopped by adding 6 *N* HCl and the non-fixed ^{14}C was released. One hundred μl of the reaction mixture were removed and placed in a scintillation vial. After drying (4 h at 60°C) the samples, 2 ml of scintillation solution (ICN, EcoLume, Costa Mesa) were added to the vial and counted in a scintillation counter (Beckman model LS 6500). The assay for PEPC was performed as above, with the following modifications: (1) buffer was added with malate dehydrogenase, (2) the assay mixture incubation was added with 10 μl NADH, and (3) the assay was started by adding 10 μl of 5 m*M* phosphoenolpyruvate.

Carboxylase polypeptides (Western blot). Total proteins in YFEL were separated by denatured SDS–PAGE gel (Laemmli, 1970). The gel was prepared with 10 or 12% polyacrylamide. The separated proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Western blot) using a mini-chamber at 200 mA, overnight at 4°C using a Bio-Rad apparatus. The Western blot analyses were used to perform semi-quantitative determinations of Rubisco and PEPC polypeptides (Santamaría et al., 1999). The polyclonal Rubisco (anti-Rubisco) or PEPC antibodies (anti-PEPC) were derived from *Nicotiana tabacum*, kindly provided by Dr. A. Nato from Université Paris IV, France. The protein was assayed with alkaline phosphatase conjugated anti-rabbit Ig-G antibody.

Growth parameters. Fresh weights of whole coconut seedlings or plantlets as well as those of leaves, stems, and roots were measured. The root to shoot (leaves + stems) ratio was calculated. Dry weights (DW) of whole seedlings or plantlets and that of plantlet parts were also measured after drying samples at 80°C until constant weight. Leaf area, number, and root number were also assessed.

Acclimatization stages and growth ex vitro. Coconut plantlets grown *in vitro* were transferred to *ex vitro* conditions, in sterile sand:soil:peat moss substrate (1:1:1 proportions) and grown in pots under fogging conditions for 2 mo. They were kept under 80–95% relative humidity, 0–120 μmol PPFD $m^{-2}s^{-1}$, photoperiod of 14/10 h (light/dark), and temperature of 27 ± 2°C. Later the plantlets were transferred to the nursery under shade conditions for an additional 2 mo. under 50–95% relative humidity, 0–600 μmol PPFD $m^{-2}s^{-1}$, photoperiod of 14/10 h (light/dark), and temperature of 18–38°C. Plantlets were finally planted in a nursery bed in full sun for an additional 40 mo. under 40–95% relative humidity, 0–2000 μmol PPFD $m^{-2}s^{-1}$, photoperiod of 14/10 h (light/dark), and temperature of 20–40°C. Plantlets were watered three times a week at 08.00 h. All parameters were tested at 11.00 h. Survival rates, photosynthesis rate, and growth were measured at the end of each acclimatization stage: *in vitro* (the end *in vitro* stage), shade (2 mo.), and full sun (28 mo.). Seedlings used as controls were grown all the time in full sun in the nursery and *in vivo* under 40–95% relative humidity, 0–2000 μmol PPFD $m^{-2}s^{-1}$, photoperiod of 14/10 h (light/dark), and temperature of 20–40°C.

Statistical analysis. One-way ANOVA test was used to analyze the data. Significant differences between means were detected by *t*-Student–Newman–Keuls test ($\alpha = 0.05$), using the statistical program Sigma Stat 1.0 for Windows (Jandel Corporation, San Rafael, CA, USA). The seedlings were not included in the statistical analysis.

Results

Daily photosynthetic rates. Six-mo.-old control seedlings showed, at first light, maximum values of A_{CO2} at 10 μmol CO_2 $m^{-2}s^{-1}$ at 11.00 h, decreasing gradually in the afternoon, showing values close to the Γ_L later in the evening (Fig. 1). Moreover, 20-mo.-old seedlings showed a similar A_{CO2} diurnal pattern.

In vitro-grown plantlets had a maximum A_{CO2} of 3.7 μmol CO_2 $m^{-2}s^{-1}$ for plantlets without sucrose, 2.8 and 3.2 μmol CO_2 $m^{-2}s^{-1}$ for plantlets grown with low (22.5 g l^{-1}) or intermediate exogenous sucrose (45 g l^{-1}). However, plantlets grown with high sucrose (90.0 g l^{-1}) showed the lowest A_{CO2}, having values of 1.5 μmol CO_2 $m^{-2}s^{-1}$, that was only 14% of those in control seedlings.

Chlorophyll content during in vitro *culture.* Control seedlings contained 42% more chlorophyll *a*, 25% more chlorophyll *b*, and 52% more carotenoids than plantlets cultured *in vitro* with 45 g l^{-1} sucrose (which showed the highest values among plantlets) (Table 1). Chlorophyll and carotenoid levels on a fresh weight basis increased as the sucrose increased from 0 to 45 g l^{-1} but decreased again with high sucrose (Table 1). There was 56% more chlorophyll *a*, 42% higher chlorophyll *a*/*b* ratio, 50% more carotenoids, and 57% higher chlorophyll plus carotenoids in plantlets cultured with 45 g l^{-1} sucrose than in those plantlets grown without sucrose.

Chlorophyll fluorescence of in vitro *plantlets.* Chlorophyll fluorescence measurements were performed to evaluate if restrictions to A_{CO2} were caused by photoinhibition. An Fv/Fm ratio of 0.8 is considered to reflect an adequate electron transport efficiency of PS II (Björkman and Demmig, 1987). Control seedlings exhibited an Fv/Fm ratio of 0.74 during the light hours, recovering at night to 0.83. Plantlets grown *in vitro* without or with low or intermediate

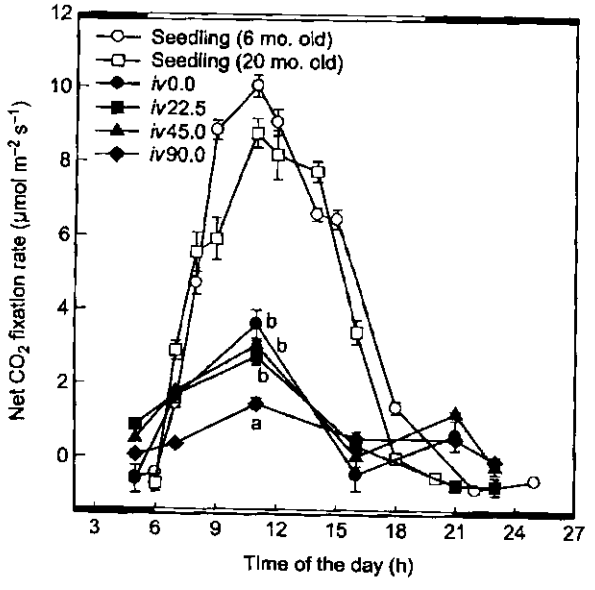

FIG. 1. Changes in net CO_2 fixation rate during a 24 h cycle. Measured on the youngest fully expanded leaf of 6- or 20-mo.-old field-grown seedlings or 8-mo.-old plantlets grown *in vitro* with four concentrations of exogenous sucrose (*iv*: 0.0, 22.5, 45.0, 90.0 $g l^{-1}$). The *upper bar* represents natural light photoperiod in the field while the *lower bar* represents the growth-room photoperiod. *Bars* are means ± SE of 10 seedlings or plantlets. Values associated with different *letters* are significantly different at $\alpha = 0.05$.

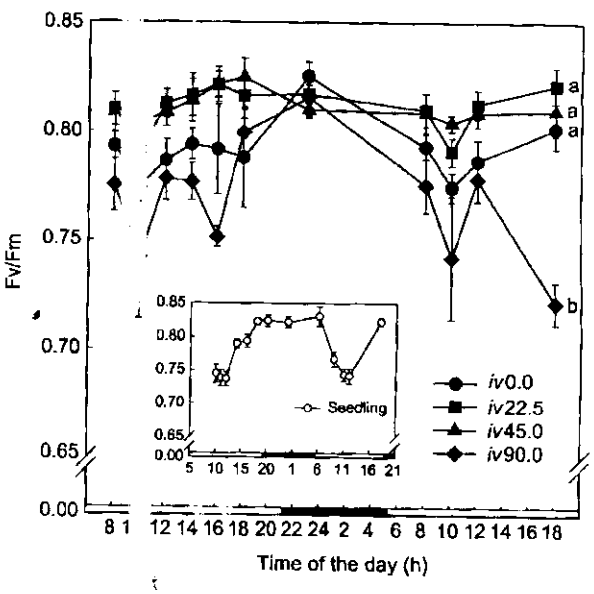

FIG. 2. Chan in chlorophyll efficiency rates of PSII, Fv/Fm, during a 24 h cycle. Mea d on the youngest fully expanded leaf of 8-mo.-old plantlets culture *vitro* with four concentrations of exogenous sucrose (*iv*: 0.0, 22.5, 0, 90.0 $g l^{-1}$), the *bar* represents the growth-room photoperiod. In insert, the behaviour of the youngest fully expanded leaf of 20-mo.-c field-grown seedlings is shown, the *bar* represents natural light ph period. *Bars* are means ± SE of 10 seedlings or plantlets. Values sociated with different *letters* are significantly different at $\alpha = 0.05$.

exogenous sucrose did not show marked differences in Fv/Fm over time, showing higher Fv/Fm values during the cycle and indicating their PSII use was efficient (Fig. 2). Thus, these plantlets did not show photo-inhibitory impairment. Conversely, high exogenous sucrose had slightly lower values during the light hours, despite recovering at night, thus showing a slight photoinhibition. The low flow of electrons through PSII in plantlets grown with high sucrose could partly explain their low photosynthesis. Though plantlets grown under low light intensity in growth-rooms do not show high photoinhibition, they may suffer from photoinhibition when transferred *ex vitro*.

Photosynthesis in light response curves. Control seedlings and plantlets cultured *in vitro* showed a typical A_{CO2} response to increasing light intensities under non-limiting CO_2 (370 μmol mol^{-1}) (Fig. 3A). Rd increased as the sucrose increased from 0 to 45.0 $g l^{-1}$ but decreased again with high sucrose, reflecting the degree of carbohydrate-induced damage of the photosynthetic system. Γ_L values were lower (3–12 μmol PPFD $m^{-2} s^{-1}$) for *in vitro* plantlets than in control seedlings (21 μmol PPFD $m^{-2} s^{-1}$) and comparable to va ues found in shaded plants. The initial slope in response to light creased with increased irradiance, indicating that electron transpor as not decreased with the low light in the growth-room, except in ntlets grown with high sucrose. Amax of control seedlings was re ed at a light intensity of about 133 μmol PPFD $m^{-2} s^{-1}$, while *i tro* plantlets reached their Amax at much lower levels (14–40 μ PPFD $m^{-2} s^{-1}$).

Photosynthesis C_i response curves. By measuring A/C_i curves on plantlets, we ere able to distinguish some of the different

TABLE 1

CONCENTRATION OF PIGMENTS (mg g^{-1} FRESH WEIGHT) FOUND IN LEAVES FROM 6-MO.-OLD COCONUT SEEDLINGS OR PLANTLETS GROWN *IN VITRO* WITH DIFFERENT SUCROSE CONCENTRATION FOR 8 MO

Pigments	Seedlings (field)	*In vitro* plantlets			
		Sucrose concentration ($g l^{-1}$)			
		0	22.5	45	90
Chlorophyll *a*	1.70 ± 0.10	0.27 ± 0.03 a	0.46 ± 0.0 b	0.60 ± 0.06 c	0.44 ± 0.02 b
Chlorophyll *b*	0.40 ± 0.02	0.16 ± 0.02 a	0.16 ± 0.0 a	0.21 ± 0.01 a	0.16 ± 0.01 a
Chlorophyll *a/b*	3.90 ± 0.06	1.70 ± 0.42 a	2.84 ± 0. b	3.01 ± 0.16 b	3.00 ± 0.07 b
Carotenoids	0.60 ± 0.03	0.14 ± 0.01 a	0.21 ± 0.0 b	0.30 ± 0.01 c	0.22 ± 0.00 b
Chlorophylls plus carotenoids	2.80 ± 0.15	0.57 ± 0.01 a	0.82 ± 0.0	1.19 ± 0.06 c	0.83 ± 0.02 b

Values followed by different *letters* within *rows* are significantly different; seedlings were not includ in the analysis.

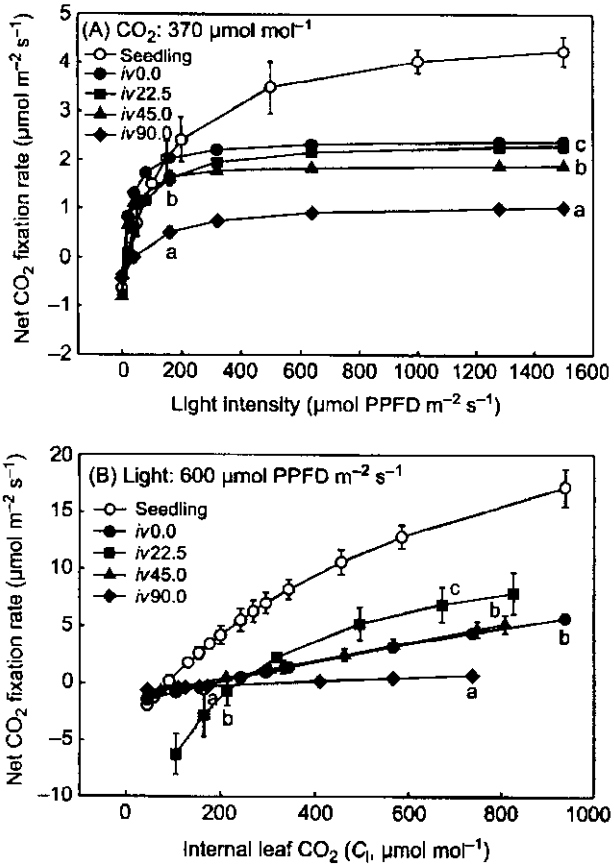

FIG. 3. Changes in net CO_2 fixation rate with increasing light intensity (*A*), and internal leaf CO_2 (*B*) of youngest fully expanded leaf from 20-mo.-old field-grown seedlings or 8-mo.-old plantlets grown *in vitro* with four concentrations of exogenous sucrose (*iv*: 0.0, 22.5, 45.0, 90.0 g l^{-1}). *A*, Curves were performed at 370 µmol CO_2 mol^{-1}; *B*, A/C_i relationship measured at 600 µmol PPFD m^{-2} s^{-1}. *Bars* are means ± SE of 10 seedlings or plantlets. Values associated with different *letters* at a given *x*-axis point are significantly different at $\alpha = 0.05$.

biochemical processes affecting the overall photosynthesis. Elevating CO_2 concentration in the air suppresses oxygenation reaction of Rubisco and increases photosynthesis of C_3 plants. Field-grown seedlings and plantlets displayed a typical A_{CO2} response to increasing C_i under a constant light intensity of 600 µmol PPFD m^{-2} s^{-1} (Fig. 3*B*). Γ_{CO2} was high for plantlets cultured *in vitro* but was lower in control seedlings. The Γ_{CO2} increase in those plantlets grown with high sucrose possibly was related to high photorespiration. Plantlets with low exogenous sucrose showed similar values of CE as control seedlings. These plantlets with diminished exogenous sucrose showed increased carboxylation efficiency, suggesting better capacity of Rubisco. The lower value of CE was found in those plantlets with high exogenous sucrose, suggesting their lower ability to assimilate CO_2. At high C_i the Amax of control seedlings increased about four times and caused an important increase about 2, 4, and 1.5 times the Amax of plantlets cultured without sucrose and with 22.5 or 45.0 g l^{-1} sucrose, respectively (Fig. 3*A*,*B*). Plantlets cultured with 90 g l^{-1} sucrose did not benefit from CO_2 enrichment and A_{CO2} remained low.

Rubisco activity and protein. Total Rubisco activity is considered to represent the main limitation to photosynthetic CO_2 fixation at saturating light and C_i given that the enzyme is fully activated. Plantlets grown without sucrose exhibited a Rubisco activity similar to control seedlings. Conversely, total Rubisco activity decreased 24, 35, and 80% from the values in control seedlings as the sucrose increased to 22.5, 45.0, and 90 g l^{-1} (Table 2). Rubisco activity was consistent when expressed on total soluble protein, chlorophyll content, or fresh weight basis. In plantlets grown with high exogenous sucrose the decrease in Rubisco activity was sufficient to account for the reduction of the A_{CO2}. The decrease in Rubisco activity was generally associated with a decrease in protein levels of the large subunit of Rubisco (55 kDa) (Fig. 4*A*). In the case of the high exogenous sucrose treatment, both Rubisco activity and Rubisco protein were drastically decreased.

PEPC activity and protein. Coconut field-grown seedlings had PEPC total activity of 14 µmol CO_2 h^{-1} g^{-1} TSP (Table 2). Conversely, PEPC total activity increased 13 times in plantlets grown *in vitro* without sucrose, 17 times in plantlets with low sucrose, and 2.7 times in intermediate sucrose compared to control seedlings. Only plantlets with high sucrose had lower PEPC activity than control seedlings. The pattern of PEPC activities (Table 2) corresponded closely to the pattern of PEPC (106 kDa) protein bands (Fig. 4*B*). In the case of the high exogenous sucrose treatment, both PEPC activity and PEPC protein were drastically decreased, being the lowest of all treatments.

Growth under in vitro conditions. Six mo. after germination, control seedlings with three fully expanded leaves showed a plant height of 79.6 cm, eight leaves, and a leaf area of the YFEL of 651 cm^2. They accumulated 40 times more whole plant dry weight (73.6 g, not including nut weight) than the average plantlets cultured *in vitro* of equivalent age. Among plantlets, those grown with high

TABLE 2

ACTIVITIES OF CARBOXYLATING ENZYMES (µmol CO_2 h^{-1} g^{-1} TSP) FROM 6-MO.-OLD COCONUT SEEDLINGS OR PLANTLETS GROWN *IN VITRO* WITH DIFFERENT SUCROSE CONCENTRATIONS FOR 8 MO.

		In vitro plantlets			
		Sucrose concentration (g l^{-1})			
Carboxylating enzymes	Seedlings (field)	0	22.5	45	90
Rubisco	18.2 ± 0.6	17.4 ± 0.9 a	13.9 ± 0.4 b	11.8 ± 0.5 c	3.6 ± 0.2 d
PEPC	13.8 ± 0.6	172.7 ± 2.6 b	232.2 ± 2.5 a	36.8 ± 1.2 c	3.1 ± 0.2 d

Values followed by different *letters* within *rows* are significantly different; seedlings were not included in the analysis. $n = 10$ plants.

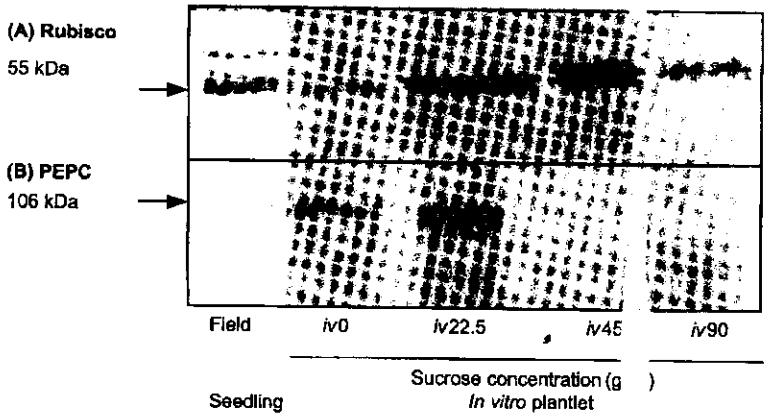

FIG. 4. Changes in Rubisco (*A*) and PEPC (*B*) proteins (Western blot). The protein was loaded om seedling control extracts (*lane seedling*) and plantlets cultured *in vitro* without (*lane iv0*), low (*lane iv22.5*), intermediate (*lane iv*), and high (*lane iv90*) exogenous sucrose in the medium ($g\,l^{-1}$). The proteins were dyed using 5-bromo-4-chloro-3-indolyl phosphat BCIP) and nitro blue tetrazolium (NBT) as substrate. The gels were loaded with 20 μg of TSP. The *arrows* indicate polypeptide bands. ne approximate size is indicated in kDa as estimated by molecular weight markers.

sucrose increased their dry weight by three times (Fig. 5*A*) and had three times higher root to shoot ratio than plantlets without sucrose.

The high root biomass and root to shoot ratio under high sucrose suggests that exogenous sucrose promotes root growth (Fig. 5*A*). Regarding plant height, the higher the sucrose up to $45.0\,g\,l^{-1}$, the taller the plantlets and the higher the number of leaves. Further increase to $90.0\,g\,l^{-1}$ resulted in a reduction in height and leaf number (Fig. 5*B*, *C*). Leaf area of the YFEL increased as sucrose concentration increased (Fig. 5*D*).

Survival rates under ex vitro *conditions.* After 8 mo. in culture, plantlets were transferred to *ex vitro* conditions under glasshouse-fogging facilities. After 4 wk in these conditions, plantlets originally grown *in vitro* without e ogenous sucrose showed only a 17% survival, while those wi 22.5, 45.0, and $90\,g\,l^{-1}$ sucrose all showed 100% plant sur val. Four weeks later, plantlets grown without sucrose did not rvive, but those with 22.5, 45.0, and $90.0\,g\,l^{-1}$ sucrose exh ited 91, 81, and 100% survival, respectively. Plantlets g n without sucrose and under low light

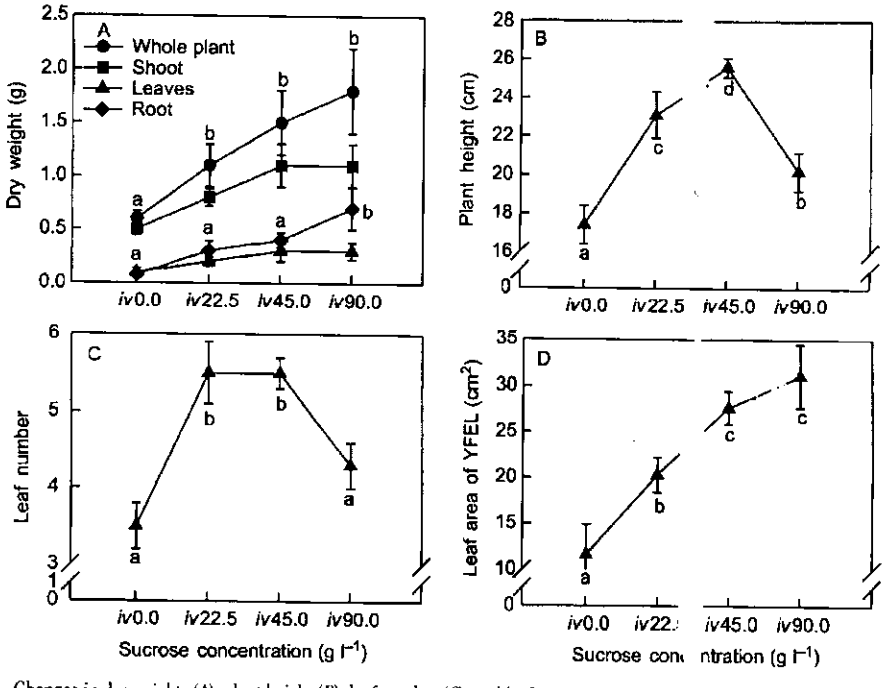

FIG. 5. Changes in dry weights (*A*), plant height (*B*), leaf number (*C*), and leaf area of YFEL (*D*). Measured from plantlets after growing *in vitro* for 8 mo. with four concentrations of exogenous sucrose (*iv*: 0.0, 22.5, 45.0, $90.0\,g\,l^{-1}$). *Bars* are means ± SE of 10 plantlets. Values associated with different *letters* for a given plant part are significantly different at $\alpha = 0.05$.

in the growth-rooms, despite having high photosynthesis, were not able to survive *ex vitro* and died. When plantlets were later transferred to the nursery under shade for a further 8 wk, plantlets originally cultured with 22.5, 45.0, or 90.0 g l^{-1} sucrose exhibited 91, 81, and 100% survival rates, respectively. At this stage, the plantlets showed the first new *ex vitro* leaf. By this time, both the *in vitro*-formed leaves and those generated *ex vitro* should contribute to autotrophic growth and survival. When plantlets were finally transferred to full-sun field conditions, the survival rates remained at 91, 81, and 100%, respectively, almost 3 yr after being transferred. Thus, survival rate is greatly decreased in the absence of sucrose and high survival can be reached even at low sucrose concentrations.

Growth under ex vitro *conditions.* Forty-four months after germination control seedlings increased 38 times their plant height (3017 cm), 19 times their leaf area of YFEL (12405 cm^2), and four times their leaf number (35 leaves). Forty-four months after being transferred *ex vitro*, plantlets grown originally with 45.0 g l^{-1} sucrose increased 78 times their plant height, 211 times their leaf area, and four times their leaf number but this represents only 66, 47, and 62% of the absolute values in the seedlings counterparts (Fig. 6*A–D*). Root to shoot ratios were higher in plantlets grown *in vitro* with sucrose than those of seedlings, but were 45 and 60% lower than those found in the same plantlets at the end of the *in vitro* stage (Fig. 6*A* versus Fig. 5*A*). Plantlets originally grown with 45.0 g l^{-1} sucrose showed the best growth of all plantlets in terms of dry weight (Fig. 6*A*), plant height (Fig. 6*B*), leaf number (Fig. 6*C*), and leaf area of YFEL (Fig. 6*D*). At both extreme conditions of low and high sucrose concentrations, plantlets showed a reduction in all the growth parameters. In plantlets grown *in vitro* with low exogenous sucrose, the A_{CO2} *ex vitro* was enough to maintain slow growth. Those plantlets grown with high sucrose, showing low *in vitro* A_{CO2}, exhibited the slowest growth (Fig. 6).

Photosynthesis rate under ex vitro *conditions.* In control seedlings grown entirely under full-sun field conditions, leaf photosynthesis was low 1 mo. after germination but rapidly increased after 3 mo., reaching a maximum A_{CO2} (9.5 μmol CO_2 m^{-2} s^{-1}) that was maintained for the next 40 mo. (Fig. 7). The noon A_{CO2} values of leaves on plantlets originally grown *in vitro* for 8 mo without, with low, or with intermediate sucrose were 3.7, 2.8, and 3.2 μmol CO_2 m^{-2} s^{-1}, respectively, while those of plantlets grown with high sucrose were only 1.5 μmol CO_2 m^{-2} s^{-1} (Fig. 7). The A_{CO2} of plantlets, except that in high sucrose, was similar to that in seedlings 1 mo. after germination. After 2 mo. *ex vitro* under fogging, plantlets with sucrose decreased their A_{CO2} except in the case of the high sucrose treatment. Four months after being transferred *ex vitro*, at the end of the shade stage, the A_{CO2} of *in vitro*-formed leaves grown under 22.5, 45.0, and 90.0 g l^{-1} sucrose increased 3.3, 9.6, and 2 times, respectively, but A_{CO2} values were still 63% lower than those of control seedlings grown in full sun. A new *ex vitro* leaf appeared after 4 mo. in all treatments. The A_{CO2} carried out by leaves formed *in vitro* could allow for autotrophic growth during the first 4 mo. of the *ex vitro* period. However, leaves formed *in vitro* never developed the same A_{CO2} as those formed *ex vitro*. After 16 mo. in full sun, leaves of plantlets originally grown *in vitro* with sucrose reached similar A_{CO2} values to control seedlings. After 30 mo., leaves of plantlets with low, intermediate, and high sucrose had A_{CO2} around 7.5, 9.0, and

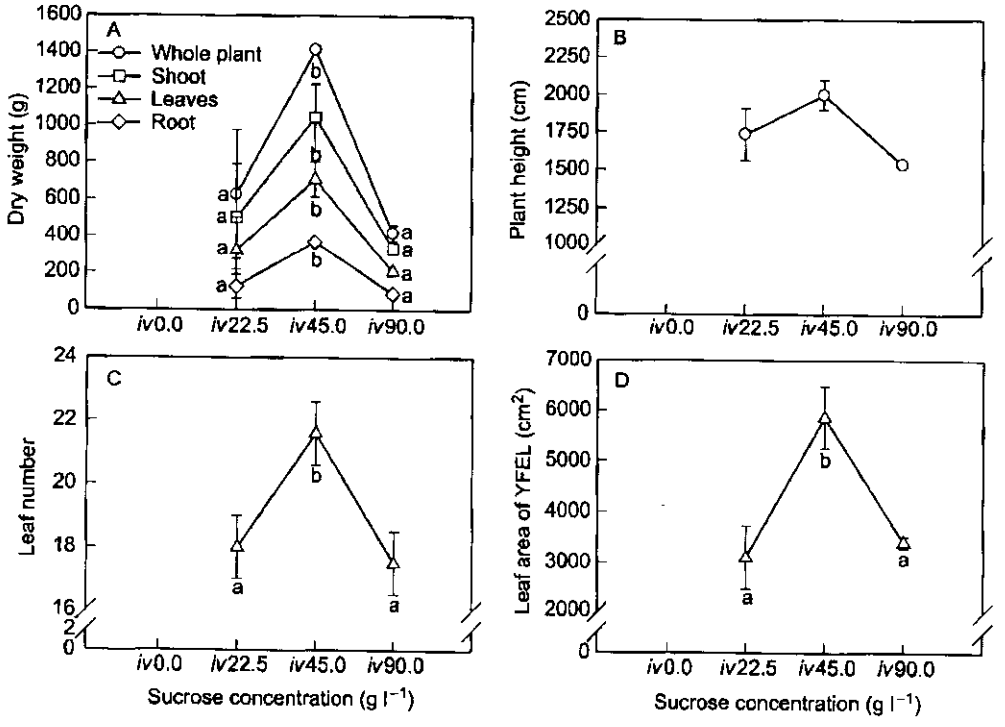

FIG. 6. Changes in dry weights (*A*), plant height (*B*), leaf number (*C*), and leaf area of YFEL (*D*). Measured from plantlets originally grown *in vitro* for 8 mo. with four concentrations of exogenous sucrose (*iv*: 0.0, 22.5, 45.0, 90.0 g l^{-1}), after 44 mo. of being transplanted *ex vitro*. Plantlets cultured without exogenous sucrose did not survive after fogging conditions. *Bars* are means ± SE of 10 plantlets. Values associated with different *letters* for a given plant part are significantly different at $\alpha = 0.05$.

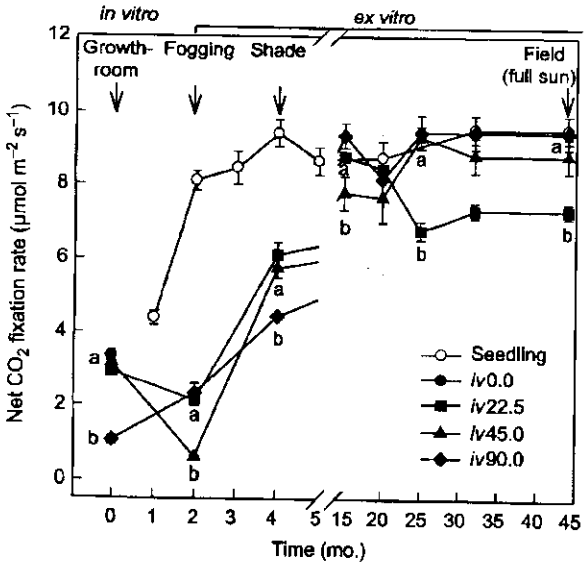

FIG. 7. Changes during 44 mo. in net CO_2 fixation rates of youngest fully expanded leaf of coconut seedlings starting 1 mo. after germination and grown all the time in full sun or of YFEL of 8-mo.-old (at the end of *in vitro* stage, time 0) coconut plantlets grown *in vitro* with four exogenous sucrose concentrations (*iv*: 0.0, 22.5, 45.0, 90.0 $g l^{-1}$). The *arrows* indicate the end of each acclimatization stage, when the plantlets were transferred from growth-room to *ex vitro* conditions: fogging, shade, and field (full sun). Plantlets cultured without exogenous sucrose did not survive after fogging conditions. *Bars* are means ± SE of five seedlings or plantlets. Values associated with different *letters* for a given time point are significantly different at $\alpha = 0.05$.

9.5 μmol CO_2 $m^{-2}s^{-1}$, respectively, that were similar to the A_{CO2} of control seedlings.

Discussion

Our data suggest that both photosynthesis and possibly reserve carbohydrates contributed to *ex vitro* performance. The similar photosynthesis found in control seedlings and plantlets clearly does not explain the very large differences in growth among them. After 44 mo. *ex vitro*, plantlets had a leaf area of 47% and dry weight of 58% of those in their equivalent control seedlings. Therefore, seedlings may have an extra source of carbohydrates, perhaps coming from seed (such as the product of β-oxidation of fatty acids in the endosperm) that are absent in the plantlets cultured *in vitro*.

Our results suggest that at least in coconut plantlets, the leaves produced *in vitro* may function not only for energy and carbon reserves but also contribute photosynthetically until new leaves could be produced *ex vitro* that would allow high photosynthetic capacity for further growth.

Effect of elimination or reduction of sucrose from the medium. Eliminating or minimizing sucrose in the medium resulted in plantlets with higher photosynthetic capacity in response to increasing light or CO_2 than in plantlets grown with higher sucrose, associated with the observed higher activity of Rubisco. However, the improved photosynthesis shown by plantlets grown without sucrose at the *in vitro* stage was not sufficient to confer a higher survival or higher growth *ex vitro* compared to their sucrose-fed counterparts. It is possible that these plantlets suffered from carbohydrate starvation, because under the low light of the standard gro -room they could show only low photosynthesis. These plantle did not have either enough carbon skeletons for *in vitro* growth nor sufficient reserves in leaves formed *in vitro* for *ex vitro* growth. These plantlets had slow root and leaf development. Additionally, in sucrose-free plantlets, the low carotenoid content could have contributed to their high susceptibility when transferred *ex vitro* to much higher light intensities.

Plantlets with low sucrose showed lower chlorophyll concentration, higher efficiency of electron transport, and higher PEPC activity and con entratio compared to the sucrose-free plantlets. They showed hi survival but slow development *ex vitro*. Therefore, low sucrose cou be enough for *in vitro* growth but those plantlets need more res ves for *ex vitro* growth that is not met by photosynthesis. both treatments plantlets were grown with low light and CO_2 o andard growth-rooms; it would be interesting to determine if suc -free or low-sucrose plantlets could show better survival and gro *ex vitro* if cultured in growth-rooms at higher light intensity o nhanced CO_2 concentrations. Experiments are currently being t ertaken in our lab exploring this possibility.

Effect of high crose in the medium. Conversely, the addition of high sucrose in the culture medium resulted in a severely diminished photosynthetic capacity of plantlets that seems to be associated with: (1) inability to respond to increasing light or C_i apparently associated with a low rate of triose phosphate utilization, perhaps as a result of decreased rates of ribulose 1,5-bisphosphate regeneration; (2) a limited electron transport to PSII as shown by low Fv/Fm values; and (3) decreased carboxylation efficiency of Rubisco and lesser mounts of Rubisco. Among these, the severe reduction in Rubi activity appears to be the limiting factor. Thus, high exogen s sucrose apparently reduced A_{CO2} by end-product inhibition. spite all the negative effects of sucrose on the photosynthetic ap atus, the added sucrose promoted root formation, good sur l, and fair growth *ex vitro* that could suggest that reserves and ot formation from exogenous sucrose are important for survi , but adequate *in vitro* photosynthesis is required for optimal owth *ex vitro*. Our results suggest that at least in coconut plantlets, e leaves produced *in vitro* may function not only for energy a l carbon reserves but also contribute photosynthetically until new leaves could be produced *ex vitro* that would allow high photosynthetic capacity for further growth.

Effect of moderate sucrose in the medium. A compromise seems to occur when an intermediate concentration of sucrose is added to the medium. Plantlets showed the highest chlorophyll and carotenoid contents of all plantlets, being in fact closer to those found in field-grown seedling controls. Additionally, they showed an adequate electron trans ort efficiency of photosystem II, and a good response of CO_2 fixatio to increasing light and C_i curves. Despite these positive effects, ey showed an intermediate efficiency and level of Rubisco protei ween both extreme conditions, although they also displayed a lo PEPC activity and protein. Plantlets grown with intermediate su se showed high survival rate, high photosynthesis rate, th stest *ex vitro* growth and the best field performance of all. general, it is suggested that the photosynthesis carried t by leaves formed *in vitro* plays an important role in suppo ng the expansion of both *ex vitro*- and *in vitro*-formed leaves and rmal growth of whole plantlets raised in medium containing sucrose (Yué et al., 1993). *In vitro*-formed leaves show transitory nutritive function, but subsequent growth could be supported solely by foliage produced *de novo* (Grout and

Millam, 1985). Our results clearly suggest that both photosynthesis and exogenous sucrose reserves contribute to *ex vitro* performance of coconut plantlets.

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