High irradiance can minimize the negative effect of exogenous sucrose on the photosynthetic capacity of *in vitro* grown coconut plantlets

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Abstract

There is increasing evidence that the sucrose normally added to the culture medium affects negatively the photosynthetic capacity of plantlets. At the same time, however, sucrose cannot be eliminated from the medium, as it is required for normal *in vitro* growth. We argue that this is true only under the conventional light conditions of growth-rooms. In the present paper irradiance of growth-rooms was increased 10 times and although the sucrose-inhibitory effect was found at high sucrose concentrations, it was possible to grow coconut (*Cocos nucifera* L.) plantlets without sucrose. Those plantlets showed both high photosynthetic capacity and comparable *in vitro* growth to those grown with sucrose in the medium under conventional growth-room irradiance. Nevertheless, the best growth was achieved under mixotrophic conditions where at high irradiance and moderate sucrose concentrations plantlets accumulated 27 % more biomass than plantlets grown without sucrose under high irradiance and 43 and 73 % more biomass than their counterparts at low irradiance with or without sucrose, respectively.

Additional key words: chlorophyll fluorescence, carboxylation efficiency, dark respiration rate, *ex vitro* transfer, light response curve, net photosynthetic rate.

Introduction

The *in vitro* culture of coconut zygotic embryos needs the addition of sugars for growth (Ashburner *et al*. 1995). However, the high sugar concentration used in the growth medium affects negatively the photosynthetic capacity of coconut plantlets (Santamaría *et al*. 1999). In a previous paper (Fuentes *et al*. 2005) it was clear that reducing the concentration of sucrose in the medium resulted in an improvement of the photosynthetic capacity but those plantlets showed a limited growth, as they have no source of carbon and photosynthesis at the irradiance of standard growth-rooms was insufficient to keep growth.

The net photosynthetic rate (P_N) in plantlets cultured *in vitro* is affected by many factors such as irradiance (PPFD), radiation quality, CO_2 concentration in the vessels, exogenous sugar type and concentrations (Jain and Babbar 2003/4). The principal storage products of carbon dioxide fixation are sucrose and starch (Goldschmidt and Huber 1992). The content of sucrose is estimated to be 10-20 times higher than other non-structural carbohydrates (Farrar and Gunn 1996). However, it has been shown that exogenous sucrose inhibits photosynthesis of *in vitro* plantlets (Hdider and Desjardins 1994). *In vitro* plantlets were at one point classified in terms of photosynthesis in two types: *in vitro*-plantlets that have a positive P_N and plantlets that show a negative P_N (Grout and Aston 1978). However,

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Abbreviations: AQE - apparent quantum efficiency; CE - carboxylation efficiency; Γ - CO_2 compensation concentration; c_i - internal leaf CO_2 concentration; F_m - maximum fluorescence; F_0 - initial fluorescence, F_v - variable fluorescence ($F_v = F_m - F_0$); $PPFD_{comp}$ - compensation irradiance; k - convexity factor; P_N - net photosynthetic rate; P_{max} - maximum photosynthetic rate; P_{sat} - photosynthetic rate at saturation irradiance; PPFD - photosynthetic photon flux density; R_D - dark respiration rate.
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the low PPFD of standard growth-rooms and the high exogenous sugar concentrations in the medium may be responsible for the low P_N in some plantlets. High biomass and sugar reserves could be critical factors for a successful acclimatization, as plantlets are able to produce new *ex vitro* organs and tissues rapidly in their new environment (Fila *et al*. 1998). *In vitro* leaves act as nutrient storage structures in acclimatization and sustained the growth of new adapted leaves *ex vitro*. Hence any attempt to increase the reserves of *in vitro* plantlets should improve acclimatization performance. Increased PPFD was found to be beneficial for plant growth in the later stages of micropropagation (Donnelly *et al*. 1985). Plantlets under low PPFD, at normal growth-rooms, limited their P_N *in vitro*, under high PPFD, however, the C availability becomes the limiting factor for photosynthesis during several hours in the light period (Navarro *et al*. 1994). PPFD plays a critical role in plant growth and development; quantity and quality are perceived by photosensory systems, which, collectively, regulate plant development, presumably to maintain photosynthetic efficiency (Hangarter 1997). Photoautotrophic micropropagation has also been shown to promote growth with better anatomical and physiological characteristics for acclimatization than conventional heterotrophic and photomixotrophic plantlets (Kozai 1991).

In the present paper plantlets were grown under a range of exogenous sucrose (0.0, 22.5, 45.0 and 90.0 g dm^{-3}) in the medium and two PPFD (40 and 400 µmol m^{-2} s^{-1}) under non limiting CO_2 concentration.

Materials and methods

Plant material and culture conditions: *In vitro* germinated embryos from coconut plantlets (*Cocos nucifera* L.) green dwarf cultivar were cultured at 16-h photoperiod under conventional PPFD (40 µmol m^{-2} s^{-1}) in liquid medium Y3 (Eeuwens 1976, modified by Rillo and Paloma 1992) for two months. Plantlets were then cultivated with four different concentrations of exogenous sucrose: 0.0, 22.5, 45.0 and 90.0 g dm^{-3} for further 8 months under two PPFD at growth-rooms: 40 µmol m^{-2} s^{-1} (standard conditions) or 400 µmol m^{-2} s^{-1}. Sub-cultures were made every two months to fresh Y3 medium. The plantlets were maintained at 26 ± 1 °C inside glass containers of 500 cm^{-3} volume containing 50 cm^{-3} medium. Coconut seedlings used as controls were germinated and grown under field (full-sun) conditions in Yucatán, México, with natural light reaching maximum of 1500 - 2000 µmol m^{-2} s^{-1} and mid-day temperatures of 35 - 40 °C.

PPFD treatments: A growth room was adapted to fit halogen lamps (*Osram*, Westfield, USA) that would allow increasing PPFD without increasing the temperature. The set up consisted in two units. One was kept at high PPFD of 400 µmol m^{-2} s^{-1} while the other unit was maintained with the same number of lamps but PPFD was lowered using two layers of black plastic mesh to 40 µmol m^{-2} s^{-1} (Fig. 1*A*). The extra heat was avoided by passing bellow the light source a film of running water to dissipate the heat. Additionally, 2 independent air conditioning units were fitted in such a way that both modules were kept at 26 ± 1 °C at all times (Fig. 1*B*). The CO_2 concentration inside both modules was kept at 1400 µmol mol^{-1} at all times and was controlled by a computerized system program developed at our Instrumentation Unit, using a PP Systems unit (*EGM-1*, Environmental Gas Monitor for CO_2, MA, USA) as a CO_2 monitor and a selenoid valve (Fig. 1*C*).

Net photosynthetic rate: Portable photosynthetic systems, *LI-6200* and *LI-6400* (*LI-COR*, Lincoln, NE, USA) were used to measure P_N of the youngest fully expanded leaf in response to increasing PPFD (from 0 to 1500 µmol m^{-2} s^{-1}) or the P_N response to increasing internal leaf CO_2 (c_i, from 0 to 1000 µmol mol^{-1}). The other 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, Dundee, UK).

Chlorophyll fluorescence: A portable *Plant Efficiency Analyzer* (*PEA*, *Hansatech*, Kings Lynn, England) was used to measure the efficiency of electron transport of photosystem 2 (PS 2) parameters. After dark-adaptation for 20 min the fluorescence parameters F_0 (minimum fluorescence) and F_m (maximum fluorescence) were measured in a 24 h cycle, and F_v/F_m (variable to maximum fluorescence ratio) was calculated.

***In vitro* growth measurements:** Fresh mass (FM) of whole coconut seedlings or plantlets as well as those of leaves, stems and roots were measured in all treatments at the end of the 8-month culture. The root to shoot (leaves + stems) ratio was calculated. Dry mass (DM) of whole seedlings or plantlets and their parts was also measured after drying samples at 80 °C to constant mass. Number of leaves and roots were also assessed.

Statistical analysis: Two-way *ANOVA* test was used to analyze the data. Significant differences between means were detected by *t*-Student-Newman-Keuls test

(α = 0.05) using *Sigma Stat 1.0 for Windows* (*Jandel Corporation*, CA, USA). The seedlings were not included in the statistical analysis. Data are presented as means ± standard error (SE) of at least 3 replicates.

Results and discussion

Response of P_N to PPFD: In all treatments, P_N increased with increasing PPFD. At low PPFD, R_D increased as the sucrose increased from 0 to 90 g dm^{-3} reflecting a high metabolic rate, except with 22.5 g dm^{-3} sucrose (Table 1). However, at high PPFD, R_D was similar without or with sucrose but was inhibited at high sucrose concentration, probably reflecting damage in those plantlets. At low PPFD, except in those plantlets at extreme sucrose concentrations (0 or 90 g dm^{-3}), plantlets had 4 times lower CO_2 compensation concentration (Γ) than those

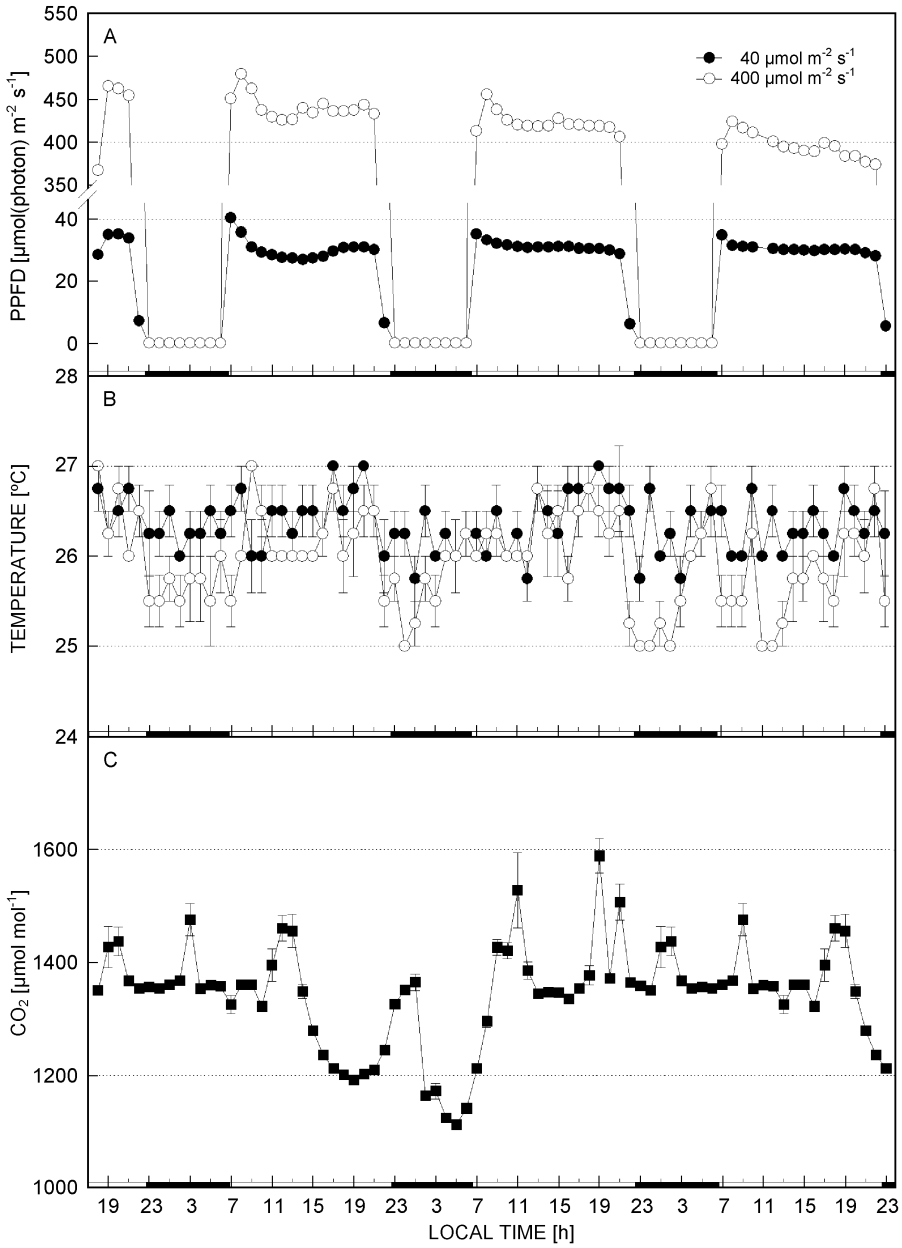

Fig. 1. Diurnal changes during 4 days in PPFD (*A*), temperature (*B*), and CO_2 (*C*) in the growth-rooms where coconut plantlets were grown *in vitro* with four exogenous sucrose concentrations at low PPFD (40 µmol m^{-2} s^{-1}, *closed symbols*) or at high PPFD (400 µmol m^{-2} s^{-1}, *open symbols*). The *lower bar* represent the growth-room photoperiod. Data are means ± SE of 10 readings.

Table 1. Values of photosynthetic parameters calculated from the curves relating P_N to PPFD. Values were obtained for six-month-old coconut seedlings or plantlets grown *in vitro* for eight months under four exogenous sucrose concentrations. Results are means ± SE of five seedlings or plantlets. Values with different letters in P_{max} are significantly different. $PPFD_{comp}$ - compensation irradiance, AQE - apparent quantum efficiency, k - convexity factor.

Sucrose [g dm^{-3}]	PPFD [µmol m^{-2} s^{-1}]	$PPFD_{comp}$ [µmol m^{-2} s^{-1}]	P_{max} [µmol m^{-2} s^{-1}]	P_{sat} [µmol m^{-2} s^{-1}]	R_D [µmol m^{-2} s^{-1}]	AQE [mol(CO_2) mol^{-1} (photon)]	k
0	40	6.0	2.82^a	179	-0.10	0.016	0.8
22.5		1.9	3.91^a	150	-0.05	0.026	0.6
45.0		15.9	3.01^a	285	-0.18	0.011	0.8
90.0		31.5	1.50^b	153	-0.40	0.013	0.0
0	400	4.3	5.28^a	101	-0.24	0.047	0.4
22.5		7.6	4.61^b	463	-0.07	0.010	0.8
45.0		65.9	4.02^b	539	-0.23	0.003	1.0
90.0		45.2	0.64^c	445	-0.07	0.001	1.0
Seedlings	1500	21.3	4.70 ± 0.31	133	-0.46 ± 0.10	0.050	0.1

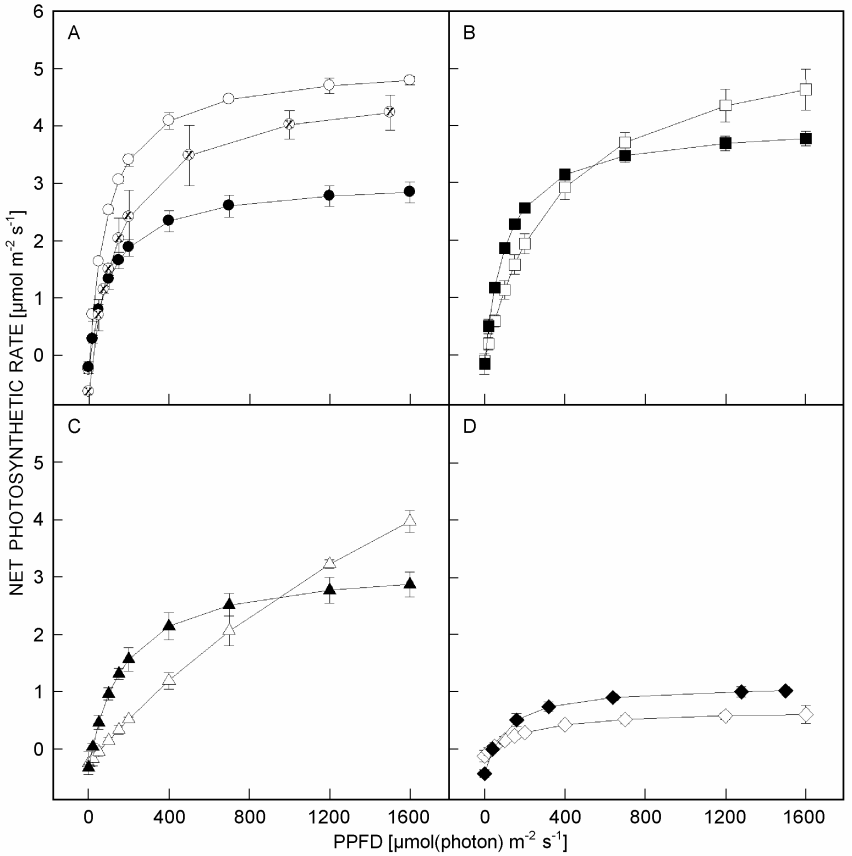

Fig. 2. Changes in net photosynthetic rate in response to increasing PPFD (light-response curves). Data are from youngest fully expanded leaf from 20 months-old field-grown seedlings or plantlets grown *in vitro* for 8 months with four exogenous sucrose concentrations (0.0, 22.5, 45.0, 90.0 g dm^{-3}) under two different PPFD: 40 (*closed symbols*) and 400 µmol m^{-2} s^{-1} (*open symbols*). The curves were performed at 370 µmol(CO_2) mol^{-1}. Data are means ± SE of 5 plantlets.

at high PPFD. The initial slope was higher in those plantlets at low than at high PPFD, probably indicating an increased light requirements for electron transport at least at high PPFD in the growth-room. At high PPFD, plantlets grown without sucrose showed the best efficiency of PPFD but it was reduced in plantlets with high exogenous sucrose. The *k* factor at high PPFD, indicated some limitation to the efficiency of the carboxylation probably on CO_2 supply to chloroplast stroma (Akhkha *et al.* 2001, Sawada *et al.* 2001). At low

PPFD, plantlets grown at 22.5 g dm^{-3} sucrose showed slightly higher photosynthetic response to increasing PPFD than those at 45.0 g dm^{-3} but high sucrose concentration inhibited photosynthetic response (Fig. 2*A-D*, *closed symbols*). However, at high PPFD, except for plantlets grown at high sucrose concentration, all plantlets had higher P_{max} than those grown at growth-room with low PPFD (Fig. 2*A-D*, *open symbols*). Increasing PPFD at growth-room, in the absence of sucrose, resulted in a dramatic increase in P_{max} from 2.8 to 5.3 µmol CO_2 m^{-2} s^{-1} (Fig. 2*A*; Table 1). In fact plantlets grown at high PPFD showed P_{max} greater than seedlings of equivalent age grown at the field. At high PPFD the P_{max} decreased as the concentration of sucrose increased (Fig. 2*A-D*). Saturation PPFD increased as concentration of sucrose increased, except in those plantlets with high exogenous sucrose. A particularly high inhibition of the photosynthetic response to increasing PPFD occurred at high sucrose concentrations with low or high PPFD (Fig. 2*D*; Table 1).

Response of P_N to c_i: At high PPFD, except for plantlets with high exogenous sucrose, R_D was lower than in those plantlets at low PPFD where all treatments showed similar R_D values. At high PPFD, plantlets showed a lower Γ than those at low PPFD, except in those plantlets with high exogenous sucrose, thus increasing fixation rates. At high PPFD, plantlets grown without or with exogenous sucrose, except those with high exogenous sucrose, showed better carboxylation efficiency than plantlets grown at low PPFD (Table 2). Again, in the absence of sucrose, plantlets grown at high PPFD had higher P_{max} than those grown at low PPFD, regardless of sucrose concentration (Fig. 3*A*). At growth-room with low PPFD, the increase in sucrose concentration caused a reduction of P_{max} (Fig. 3*A-D*, *closed symbols*).

Chlorophyll fluorescence: Except when plantlets were grown in the absence of sucrose, where F_v/F_m ratios (the efficiency of PS 2) were similar at low or high PPFD, in all other sucrose treatments plantlets grown at low PPFD showed higher F_v/F_m ratio (0.8 is for healthy plantlets, Björkman and Demmig 1987) than those grown at high PPFD perhaps as a result of photoinhibition (Fig. 4*A-D*). Under low PPFD, sucrose had little effect on F_v/F_m, plantlets showing similarly high values during the light

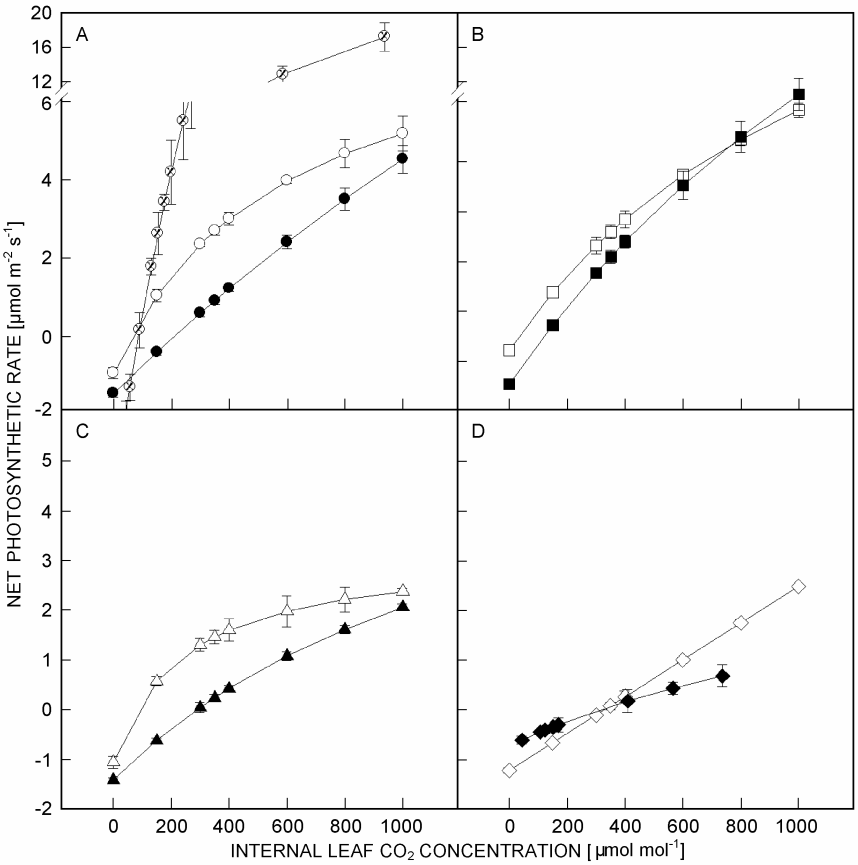

Fig. 3. Changes in net photosynthetic rate in response to increasing internal CO_2 concentration (P_N/c_i curves) of youngest fully expanded leaf from 20 months-old field-grown seedlings or plantlets grown *in vitro* for 8 months with four exogenous sucrose (0.0, 22.5, 45.0, 90.0 g dm^{-3}) under two PPFD: 40 (*closed symbols*) and 400 µmol photon m^{-2} s^{-1} (*open symbols*). P_N/c_i curves were performed at PPFD of 600 µmol m^{-2} s^{-1}. Data are means ± SE of 5 plantlets.

Table 2. Values of photosynthetic parameters calculated from the curves of relating P_N to c_i. Values were obtained for six-month-old coconut seedlings or plantlets grown *in vitro* for eight months with four exogenous sucrose concentrations. Results are means ± SE of five seedlings or plantlets. Values with different letters in P_{max} are significantly different. CE - carboxylation efficiency, Number - number of CO_2 molecules fixed.

Sucrose [g dm^{-3}]	PPFD [µmol m^{-2} s^{-1}]	Γ [µmol(CO_2) mol^{-1}]	P_{max} [µmol m^{-2} s^{-1}]	R_D [µmol m^{-2} s^{-1}]	CE [µmol m^{-2} s^{-1}]	Number
0	40	209.0	4.4^a	-1.5	0.07	14.30
22.5		188.0	4.2^a	-1.5	0.08	12.50
45.0		288.0	2.1^b	-1.4	0.06	16.70
90.0		300.0	0.5^c	-0.7	0.03	38.00
0	400	62.5	4.9^a	-1.0	0.17	5.90
22.5		96.8	3.9^b	-0.8	0.09	11.10
45.0		79.4	2.6^c	-1.0	0.17	5.90
90.0		300.0	2.5^c	-1.2	0.04	25.00
Seedlings	1500	79.0	19.7 ± 0.73	-2.3 ± 0.25	0.40	2.50

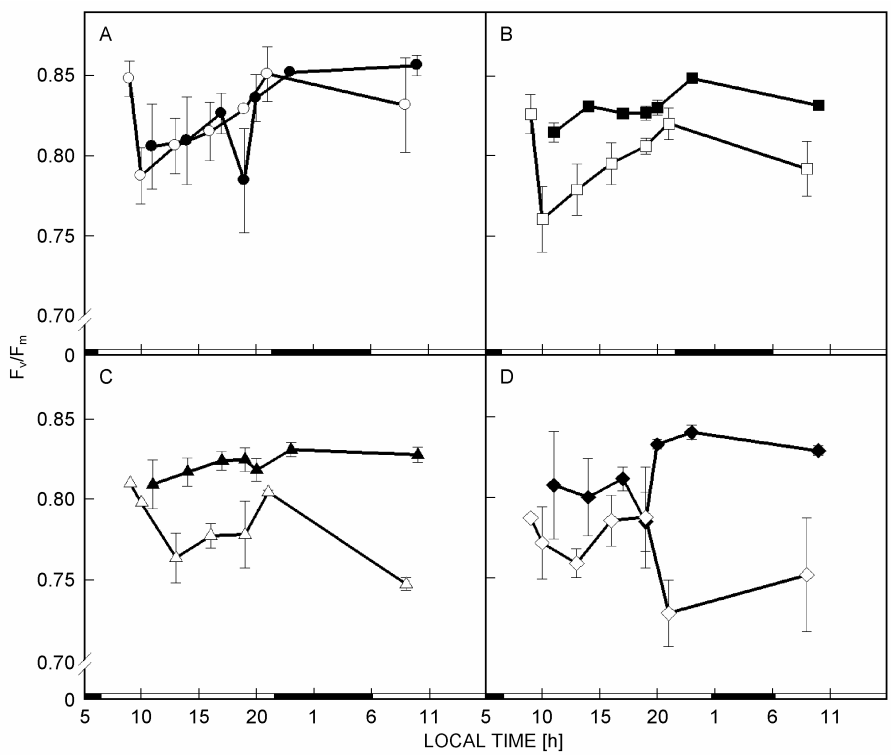

Fig. 4. Changes in efficiency of PS 2, F_v/F_m, during a 24 h cycle. Measured on the youngest fully expanded leaf of plantlets grown *in vitro* for 8 months with four exogenous sucrose concentrations (0.0, 22.5, 45.0, 90.0 g dm^{-3}) under two PPFD: 40 (*closed symbols*) and 400 µmol m^{-2} s^{-1} (*open symbols*). The *lower bar* represents the growth-room photoperiod. Data are means ± SE of 5 plantlets.

hours and increasing slightly at night (Fig. 4*A-D*, *closed symbols*). If any, under high exogenous sucrose conditions (90 g dm^{-3}) F_v/F_m ratios decreased slightly at the end of the day but recovered fully at night. At high growth-room PPFD, however, the efficiency of PS 2 decreased considerably as the sucrose from the medium increased (Fig. 4*A-D*, open symbols). In this case, F_v/F_m remained low during the light hours and did not recover at night.

Growth parameters: At low PPFD, plantlets grown with low exogenous sucrose were the tallest of all plantlets, while those with very high exogenous sucrose were the shortest (Fig. 5*A*). Plantlets at high PPFD showed slightly lower height. Plantlets grown at high PPFD showed more leaves than those grown at low PPFD independently of sucrose concentration (Fig. 5*B*). Under low PPFD plantlets cultured at extreme concentration (0 or 90 g dm^{-3} sucrose) showed lower number of leaves than those plantlets grown with 22.5 or 45.0 g dm^{-3} sucrose. Total plant fresh and dry masses were higher in plantlets at

high PPFD than those at low PPFD irrespective of their exogenous sucrose concentrations in the medium (Fig. 5*C*). In the absence of sucrose, at both PPFD treatments plantlets showed slightly lower biomass accumulation than those plantlets grown with exogenous sucrose in the medium. However, those plantlets at high PPFD grown without sucrose showed higher dry mass than the best treatment at low PPFD. Plantlets at high PPFD had higher shoot or root masses than those at low PPFD irrespective of their sucrose concentration treatments (Fig. 5*D*,*E*). Plantlets at high PPFD had 2 times more root dry mass than those at low PPFD (Fig. 5*E*). On the contrary, the root to shoot ratio was 86 % higher in those plantlets with sucrose than those grown without sucrose at low PPFD and 72 % higher under high PPFD. Plantlets at low PPFD had higher root to shoot ratio than those at high PPFD almost at all sucrose concentrations (Fig. 5*F*). Exogenous sucrose promoted faster root formation and high root to shoot ratios at both PPFD. Low PPFD had an important effect on plant dry mass but this was more pronounced in those plantlets without exogenous sucrose.

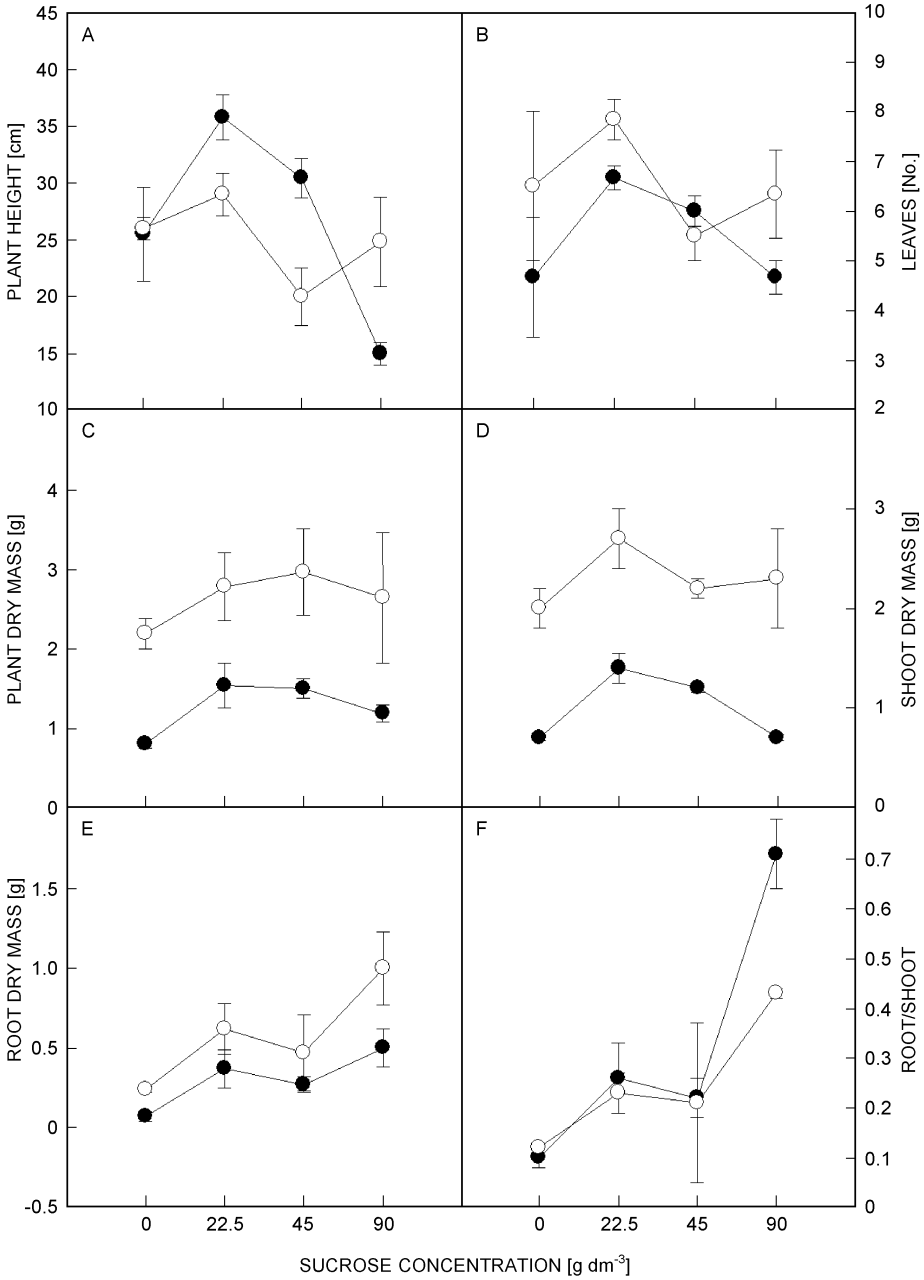

Fig. 5. Growth parameters: plants height (*A*), leaf number (*B*), plant dry mass (*C*), shoot dry mass (*D*), root dry mass (*E*), root/shoot ratio (*F*), of plantlets grown *in vitro* for 8 months with four exogenous sucrose concentrations (0.0, 22.5, 45.0, 90.0 g dm^{-3}) and under two different PPFD: 40 (*closed symbols*) and 400 µmol m^{-2} s^{-1} (*open symbols*). Means ± SE of 5 plantlets.

Effect of PPFD: On average, plantlets grown at high PPFD clearly showed a better photosynthetic response to increasing PPFD and c_i, higher leaf number and plant dry mass. However, plantlets grown at low PPFD showed better electron transport efficiency to PS 2 than those plantlets at high PPFD, indicating some degree of photoinhibition in those plantlets. However, those plantlets grown at high PPFD, could increase their electron transport efficiency as well as photoprotective responses such as thermal energy dissipation when later transferred to *ex vitro* conditions. Tomato *in vitro* plantlets grown at high PPFD with sucrose showed high F_v/F_m ratios (Le Van *et al*. 2001). Wheat plants under high PPFD and subsequently exposed to very high PPFD suffered a less high irradiance stress and photo-oxidative degradation of pigment compared with those grown under low PPFD (Behera and Choudhury 2002).

On average, our plantlets grew better at high PPFD than those at low PPFD. Similar results have been shown for other species (Karim *et al*. 2003, Sha Valli Khan *et al*. 2003). Moreover, plantlets grown at high PPFD, could eventually show faster recovery, specially at short time after they are transferred to *ex vitro* conditions thus, light plays a decisive role in the development of the photosynthesis capacity and growth of coconut plantlets.

Effect of exogenous sucrose concentration: Significant differences in those plantlets with different sucrose treatments were found in most photosynthetic and growth parameters. Plantlets grown without exogenous sucrose or those with low (22.5 g dm^{-3}) showed a better PS 2 efficiency, photosynthetic response to increasing PPFD or c_i and growth. In fact the increase in sucrose concentration decreased the slope of the PPFD response curves. The negative effects observed in plantlets with high sucrose may be related to an increased plant susceptibility to feedback inhibition, which is possibly associated to excessive accumulation of hexoses and starch (Le *et al*. 2001). Our results showed that the high exogenous sucrose concentration caused an increase in stems and root dry mass but a decrease in plant height or leaf number and dry mass resulting in increased root to shoot ratios. Similar results have been shown for other species (Morini and Melai 2003/4). It was clear that sucrose diminished P_N but increased dry biomass, thus low exogenous sucrose can be used to improve carbon fixation and *in vitro* or even *ex vitro* growth. Exogenous sucrose plays a role in resource allocation patterns of plantlets.

Combined effects of PPFD and exogenous sucrose: Plantlets grown without exogenous sucrose irrespective of PPFD, certainly had increased photosynthetic capacity, then the source-sink equilibrium was shifted towards a source limitation with positive effects on shoot growth. On the other hand, plantlets grown with high exogenous sucrose irrespective of PPFD, certainly had limited photosynthetic capacity, then the source-sink equilibrium was shifted towards a sink limitation with positive effects on root growth. In plantlets without exogenous sucrose at high PPFD, the sucrose effect on photosynthetic capacity was overcome while at low PPFD the effect of sucrose was not substantially affected. Those plantlets at high PPFD but with high exogenous sucrose showed negative effects on growth and P_N. Similar results were obtained for *in vitro* plantlets of tomato (Le *et al*. 2001). Increase in dry mass as a result of exogenous sucrose has been reported earlier (Abdin *et al*. 1998). When plantlets at low PPFD were supplied with sucrose, the growth was good but never reached the growth obtained at high PPFD without sucrose. Reductions in dry matter due to low PPFD were more pronounced for leaves and stems than for roots.

Thus, although exogenous sucrose provides an alternative source of carbon in plantlets grown *in vitro*, it is not large enough to overcome the negative effects of low PPFD of standard growth-rooms on plant biomass accumulation and morphology. This study has provided evidence of a decoupling relation between light and sucrose on photosynthesis and other light processes that control plant responses. It is possible that available exogenous sucrose is less important to morphological plant responses than PPFD. Thus, the addition of high exogenous sucrose does not substitute the beneficial effects of high PPFD on dry mass.

Despite the presence of substantial levels of exogenously supplied sucrose, PPFD played a key role in the development and morphology of the plantlets that have relation with growth (increase in biomass). In this study, plantlets at high PPFD without sucrose in the medium produced greater biomass (23 %) than those plantlets grown at low PPFD even with sucrose, suggesting that sufficiently high photosynthesis can substitute the need to add sucrose for growth. At low PPFD, plantlets grown with low sucrose, increased 53 % on average, their total plant dry mass relative to those without sucrose.

Increased PPFD stimulates better growth than does the addition of exogenous sucrose, which may have negative effects on photosynthesis. These results demonstrate that *in vitro* coconut plantlets had the best growth and P_N when they are grown at high PPFD and low exogenous sucrose. Experiments are needed to define if coconut plantlets grown without sucrose at higher PPFD than those in conventional growth-rooms, are able to survive the transfer *ex vitro* and grow better at the nursery. As mentioned earlier, other authors have suggested that the main limitation for *ex vitro* growth, in plantlets cultured *in vitro*, is their low photosynthetic capacity (Arigita *et al*. 2002).

The higher biomass in those *in vitro* plantlets grown under high PPFD at growth-room, possibly indicates, that this specie could grow better under greenhouse conditions with high PPFD from natural light than under standard

growth-room conditions. In banana, other tropical specie, plantlets showed higher multiplications rates under non-controlled natural light conditions than under artificial light in the growth chambers (Kodym and Zapata-Arias 1999). Tropical species cultured *in vitro* need to adapt to *ex vitro* conditions showing very high PPFD and temperature. Greenhouse-like culture rooms under tropical conditions could be the next step for growing coconut *in vitro* plantlets or other tropical species with higher photosynthesis and better chances of showing high survival and normal growth when transferred to the field.

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