Effect of medium sucrose on the photosynthetic capacity of coconut vitroplants formed from zygotic embryos

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1. Introduction

The coconut palm is an important source of oil and other products in tropical countries. Coconut populations in the Caribbean area, including Florida (USA), México, Belize and Honduras have being lowered by a severe disease called lethal yellowing (LY) that has killed millions of plants (Oropeza and Zizumbo, 1997). In order to control this disease it is necessary to implement plant-breeding programs to increase both LY resistance and yields. For a successful breeding program it is important to have safe and efficient ways to exchange plant material from country to country. It is clear that the *in vitro* culture of zygotic embryos meets the need for phytosanitary requirements for plant material transport from country to country. However, the acclimatisation of plants derived from tissue cultured zygotic embryos is not effective enough and shows poor survival rates (Ashburner *et al.*, 1994). It is important to study the physiology of these vitroplants to determine if the problem is related to problems in controlling water loss when vitroplants are transferred from the *in vitro* container to the field or to problems with the development of photosynthetic ability.

Previous studies in coconut have shown severe reductions in photosynthetic rates, chlorophyll concentrations, activity and amount of Ribulose 1,5-biphosphate carboxylase-oxygenase (RubisCO, EC 4.1.1.39) in vitroplants derived from zygotic embryos when compared to greenhouse acclimatised palms (Triques *et al.*, 1997a; 1997b). It is likely that these reductions might be even larger when compared to values from field-grown plants. It is possible that the reduction in the

activity of RubisCO could be related to the fact that these vitroplants receive large amounts of sugars in the medium (60 g l^{-1}). Hdider and Desjardins (1994), have shown that the activity of RubisCO can be affected by sucrose in strawberry vitroplants. The objective of the present study was to determine if sucrose in the medium can be responsible for a low photosynthetic ability in vitroplants derived from coconut zygotic embryos cultured *in vitro*.

2. Materials and Methods

Plant material and culture conditions.- Nuts from *Cocos nucifera L.* adult palms were collected from green Malayan Dwarf plantations in Yucatán, México. The embryos were dissected from the nuts and cultured *in vitro* in Eeuwens'(1976) medium, as modified by Rillo and Paloma (1992). Culture conditions in the growth rooms were as follows: darkness during embryo germination and later, a photoperiod 16/8 h with room temperatures of around 27°C and light intensity (photon flux densities) of 45-60 µmol $m^{-2}s^{-1}$ (PAR).

Experimental approach.- Embryos were cultured in Y3 liquid medium supplemented with 45 g l^{-1} of sucrose for twenty weeks (subculturing every 4 weeks). Plants were then transferred to Y3 liquid media containing either a) no sucrose, or b) 45 g l^{-1} sucrose. Plants were analysed after 10 weeks of culture in either of these two treatments, when vitroplants showed from 1 to 3 bifurcated leaves and secondary roots. For analysis, leaves were dissected into three sections: base (B), middle part (M) and tip (T). These leaf sections were chosen because they show an increasing pigmentation gradient. Roots were not included in the analysis. All data presented are means from three replicates.

Histology.- Fresh leaf tissues were fixed in glutaraldehyde 2% in 0.2 M phosphate buffer for at least 2h and post-fixed in 1% osmium tetroxide for 3h. Samples were dehydrated in ethanol at progressive dilutions (10, 30, 70, 80, 90 %) before being placed in absolute ethanol. Samples were infiltrated and embedded in Spurr's resin. Ultrathin sections were observed in a TEM at 70 Kv. Sections of 3.5 µm were used for observations in optical microscope before being stained with Schiff's reactive and naphthol blue black. Slides were mounted with Surgipath and 30 observations per treatment were made.

Pigment concentration.- Fresh tissues were extracted at 4°C in the dark in 80% (v/v) acetone. Pigments were quantified spectrophotometrically and calculations performed as described in Litchtenhaler and Wellburn (1983).

Total soluble protein.- Total soluble proteins were determined in crude extracts and quantified spectrophotometrically at 610 μm according to Sedmak and Grossberg (1977).

Activities of carboxylases.- Crude enzymatic extracts were prepared form samples of fresh leaves, according to Nato and Mathieu (1978). PVP (33%; w/w) was added during the extraction to prevent polyphenolic compounds from forming, according to Rival *et al* (1997). Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and RubisCO (EC 4.1.1.39) activities were assayed following the incorporation of ^{14}C-labelled sodium bicarbonate into acid stable compounds as described in Nato *et al.* (1985). The incubation time was 15 minutes at 30° C.

Quantification of carboxylases.- Semiquantitative determinations of RubisCO and PEPC were performed by electrotransfering the products of electrophoresis from PAGE-SDS gels to PVDF membranes using semidry western blots. Polyclonal antibodies raised against the 2 carboxylases purified from tobacco leaves (RubisCO) and from maize and tobacco leaves (PEPC) were used for specific detection. Each well in the gel was loaded with the same amount of TSP (50 μg for PEPC and 0.5 μg for RubisCO assays) for each treatment.

Table 1. Anatomical characteristics of leaves (tip sections) from zygotic embryo-derived coconut vitroplants cultivated under two different sucrose treatments.

Sucrose g l^{-1}	*No. of cell layers*	*No. of layers of mesophyll cells*	*No. of chloroplasts*	*No. of starch granules/ chloroplast*
0	7.80 ± 0.76	4.27 ± 0.69	4.37 ± 0.72	0.00 ± 0.00
45	7.87 ± 0.57	4.07 ± 0.64	5.07 ± 1.01	2.83 ± 1.44
*F	0.28	4.99	6.9	42.9
p	0.74	0.008*	0.001**	0.0001**

Data are means ± SD of 30 observations per treatment. (*) Results from analysis of variance (ANOVA); (F) Snedecor's estimate, and (p) type 1 error for the effect of sucrose.

3. Results

3.1. Leaf anatomical characteristics

The effect of sucrose on leaf (tip section) anatomy is summarised in Table 1. The number of cell layers or the number of layers of mesophyll cells, were not

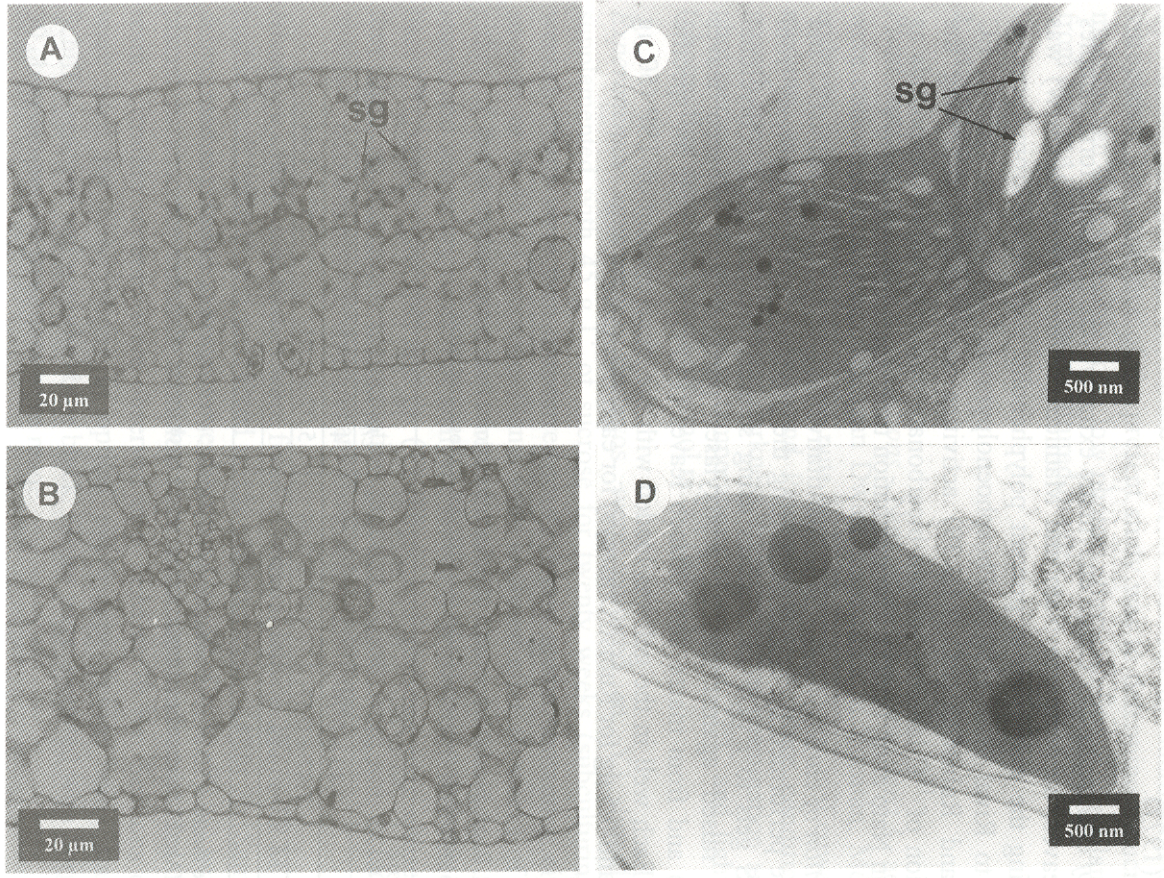

Figure 1. Optical micrographs from leaves of coconut vitroplants grown with 45 g l^{-1} sucrose (A) or without sucrose in the medium (B). Electron micrographs of mesophyll cell chloroplasts from leaves of coconut vitroplants grown with 45 g l^{-1} sucrose (C) or without sucrose in the medium (D). Note differences in the amount of starch granules [sg].

statistically different between the two treatments. On the other hand, the plants grown without sucrose in the medium showed less chloroplasts and less starch grains in the mesophyll cells than those grown with 45 g l^{-1} sucrose. These features can also be observed in Figure 1A and B at the histological level, and in Figure 1C and D at the ultrastructural level.

3.2. Pigments concentrations

The effect of sucrose in the medium on the concentrations and ratios of pigments in various tissues is shown in Table 2. In middle part leaf tissues, the concentration of total chlorophylls was similar for both treatments. In tip leaf tissues, the concentration of total chlorophylls decreased significantly when plants were grown without sucrose compared to those grown with 45 g l^{-1} of sucrose in the medium. The chlorophyll a/b ratios remained similar in both conditions.

Table 2. Concentrations of pigments, and chlorophyll a/b ratios in middle part (M), and tip (T) leaf tissues from plants grown under two different sucrose concentrations in the medium.

Sucrose g l^{-1}	*Tissue*	*Chlorophyll a mg g^{-1} FW*	*Chlorophyll b mg g^{-1} FW*	*Carotenoids mg g^{-1} FW*	*Total chloro-phyll (a + b)*	*Chl a / chl b ratio*
0	T	0.22 ± 0.10	0.12 ± 0.07	0.05 ± 0.008	0.3 ± 0.04	1.8 ± 0.7
45	T	0.31 ± 0.17	0.28 ± 0.28	0.08 ± 0.06	0.6 ± 0.50	1.1 ± 0.9
0	M	0.06 ± 0.01	0.04 ± 0.02	0.05 ± 0.01	0.1 ± 0.04	1.5 ± 0.6
45	M	0.07 ± 0.02	0.03 ± 0.02	0.07 ± 0.02	0.1 ± 0.10	2.3 ± 0.4
*F		0.6 (s) 11.7(t)	0.6(s) 0.74(t)	1.64(s) 0.03(t)	5.5(s) 0.68(t)	0.15(s) 0.38(t)
p		0.42(s) 0.009(t)	0.45(s) 0.13(t)	0.23(s) 0.87(t)	0.04*(s) 0.43(t)	0.71(s) 0.54(t)

Values are the means ± S.D. from 3 independent experiments. (*) Results from analysis of variance (ANOVA); Snedecor's estimate (F) and type 1 error (p) for the effect of sucrose (s) and for the effect of tissue (t).

3.3. Total soluble protein

Leaves from zygotic-embryo-derived vitroplants grown in the sucrose-containing medium, showed a reduced concentration of total soluble protein (TSP) (3.1 mg g^{-1} FW) when compared to those grown in sucrose-free media (4.5 mg g^{-1} FW). This difference was particularly drastic in tissues from the leaf middle part (1.6 vs. 2.4

mg g^{-1} FW). Tissues from the base of the leaves showed less TSP (1 mg g^{-1} FW) than leaves but sucrose had no effect in the concentration of TSP. As a result, on average there was not a statistically significant effect of sucrose on TSP but a highly significant effect of the tissue type (data not shown).

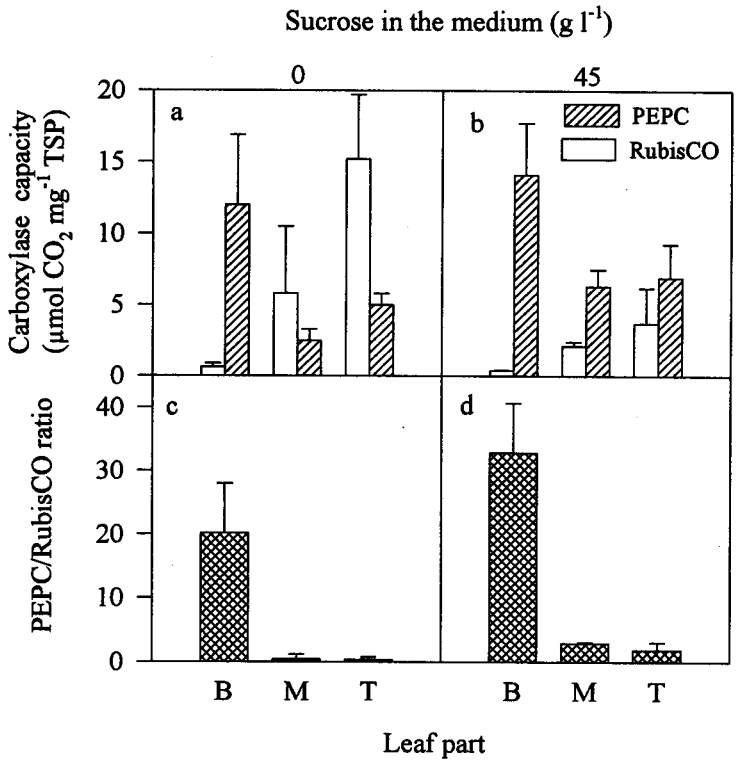

Figure 2. PEPC and RubisCO capacities (a and b) and PEPC/RubisCO ratios (c and d) in different leaf parts (base [B], middle [M] and tip [T]) of coconut vitroplants grown in 0 g l^{-1} sucrose (a and c) and 45 g l^{-1} sucrose (b and d). Bars are means and SD of 3 replicates.

3.4. Carboxylases activities

The effect of removing sucrose from the medium on the carboxylases activities of various tissues is shown in Figure 2. In general, Rubisco activities were very low in tissues from the base of the leaves, relative to tissues in the middle and tip parts of the leaves in both sucrose treatments. The removal of sucrose increased

significantly the activity of RubisCO in all tissues. In tissues at the middle part of the leaf from plants grown with sucrose, the RubisCO capacities were only 66% while in older leaf tissues of the leaves they were only 24% of those of plants grown in sucrose-free media. On the other hand, PEPC activities were high in tissues from the base of the leaves and lower in the middle and tips. The removal of sucrose, although on average, had no significant effect on PEPC activities, decreased the PEPC activities in leaf tissues (152% in M and 38% in T). The PEPC/RubisCO ratios were higher in the base of the leaves than in tissues from leaf middle or tips parts. The removal of sucrose from the medium decreased significantly the PEPC/RubisCO ratios in all tissues (725% in M, 600% in T and 163% in B).

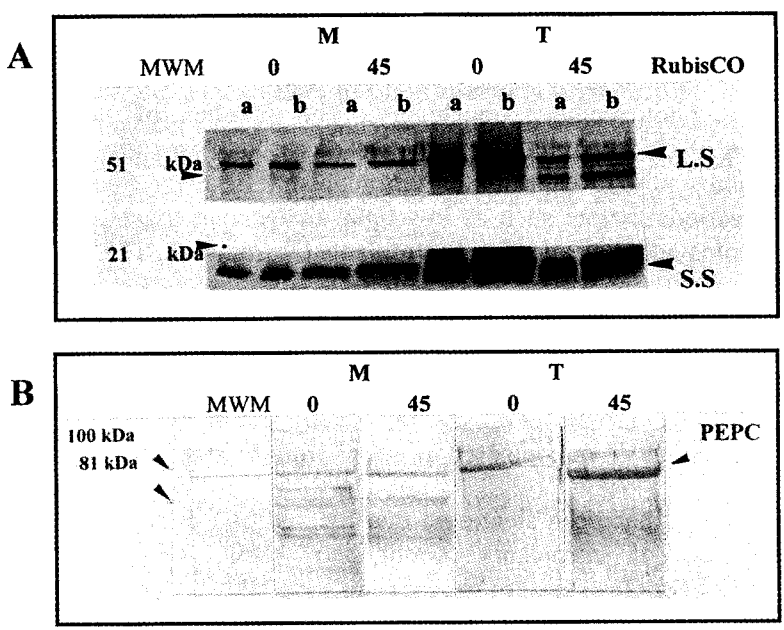

Figure 3. (A) Immunoblots from the middle [M] and tip [T] parts of leaves of plantlets cultured in medium without sucrose [0] or in medium with 45 g l^{-1} [45] sucrose, loading the wells with 0.25 μg [a] and 0.5 μg [b] of TSP. Gels were electrotransferred to PVDF membranes treated with specific polyclonal antibody raised against the large subunit [LS] and the small subunit [SS] of RubisCO purified from tobacco leaves. (B) Immunoblots from the middle [M] and tip [T] parts of leaves of plantlets cultured in medium without sucrose [0] or in medium with 45 g l^{-1} [45] sucrose. The wells were loaded with 30 μg of TSP and electrotransferred to PVDF membranes treated with specific polyclonal antibody raised against PEPC purified from maize and tobacco leaves.

3.5. Relative concentrations of carboxylases

Relative concentration of RubisCO.- Immunoblots revealed that sucrose in the medium decreases the quantity of the large and the small subunits of RubisCO (Fig. 3A). In old leaf tissues from plants grown in sucrose-containing medium, some products of degradation of the large subunit (with slightly lower molecular weight) of RubisCO which were recognised by the antibody can also be observed. In tissue from the base of the leaves, no RubisCO was detected in any treatment.

Relative concentration of PEPC.- Opposite from the effect of sucrose on the concentration of RubisCO, the concentration of PEPC in tissues grown in sucrose-containing medium was increased, particularly in leaf tip tissues (Fig. 3B).

4. Discussion

The photosynthetic capacity of a plant is the result of the integration of various parts of the photosynthetic machinery. The number of chloroplasts, the concentration of chlorophylls, the activities of carboxylases are all important in the final photosynthetic capacity of vitroplants. It is clear that under *in vitro* conditions, various factors such as low light, exogenous sugars, low CO_2 might affect the photosynthetic capacity of vitroplants (Kozai *et al.*, 1990).

Previous studies in coconut vitroplants have shown severe reductions in photosynthetic rates, chlorophyll concentrations, activity and amount of RubisCO in vitroplants derived from zygotic embryos when compared to greenhouse-acclimatised palms (Triques *et al.*, 1997a; 1997b). It is likely that these reductions might be even larger when compared to values from field-grown plants.

For a long time, it has been suggested that the addition of sugars is important in the development of vitroplants (Grout and Aston, 1978). However, high concentrations of sucrose used in the medium have shown to be detrimental in the development of photosynthetic capacity and later acclimatisation ability of vitroplants (Kozai *et al.* 1990; Kozai 1991), particularly in terms of carboxylases efficiency (Hdider and Desjardins 1994; 1995). It was therefore expected that the Rubisco activity of coconut vitroplants will improve if sucrose was removed from the medium, but the possible effect on other photosynthetic parameters was unknown. Our results suggest that the reduction of sucrose from the medium reduced slightly the number of chloroplast per mesophyll cell, however, those chloroplasts show less starch granules. Similarly, the reduction of sucrose in the

medium resulted in a decreased chlorophyll concentration in older leaf tissues. Serret *et al.* (1996) also found that the reduction of sucrose from 3% to 0.5% resulted in a reduced chlorophyll content of Gardenia vitroplants.

On the other hand, the removal of sucrose from the medium resulted in plants showing increased Rubisco activities and slightly reduced PEPC activities. In strawberry cultures sucrose also inhibited RubisCO activities while promoting PEPC activities (Hdider and Desjardins, 1994; 1995). Coconut is a C_3 plant and therefore we expected to have negligible PEPC activities, however it is a common feature to find high PEPC activities in C_3 plants of various species grown *in vitro* particularly in early stages and then get reduced as the vitroplants grow older (Desjardins, 1995). The PEPC/RubisCO ratio has been used as an indicator of the degree of autotrophy of vitroplants (Desjardins, 1995) as it reflects the relative proportion of anaplerotic to photosynthetic CO_2 fixation. The smaller the PEPC/RubisCO ratio, the more autotrophic the vitroplant. From our results showing that coconut vitroplants growing without sucrose in the medium showed smaller PEPC/RubisCO ratios than those grown with high sucrose in the medium, it can be stated that coconut vitroplants would increase their degree of autotrophy by decreasing the sucrose in the medium. It should now be studied whether this increased autotrophy would result in better survival and better plant quality when vitroplants get transferred to field conditions.

One contribution of the present paper is that using immunological techniques, it was shown that the plants grown in sucrose-containing medium that showed decreased RubisCO activity, also showed a decreased quantity of the large subunit of RubisCO presumably due to some kind of degradation of the carboxylase. The reason for the degradation of the large subunit of RubisCO in plants grown with high sucrose in the medium is unknown and deserves further investigation. It is possible that the low Rubisco activity found in earlier reports in coconut leaves from plantlets derived from zygotic embryos (Triques *et al.*, 1997a; 1997b; Rival *et al.*, this volume) could be explained by a reduction in the concentration of the carboxylase due to the high concentration of sucrose (60 g l^{-1}) that was added to the medium.

Clearly, further research is still to be done in order to clarify whether these effects of sucrose on both activities and relative amounts of RubisCO and PEPC, are related specifically to sucrose or the effects are related to the concomitant increase in the osmotic potential in the medium. Additionally, if it can be demonstrated that the observed effect is not an osmotic effect and it is particularly related to sucrose,

it will then be important to define if other sources of sugars, such as glucose or fructose, are also signals for the observed effects on the carboxylases.

The findings reported here, suggest that while sucrose might be important in early stages of coconut embryo cultures, to keep high chlorophyll concentrations and high number of chloroplasts. The continuous growth of the resulting vitroplantlets in sucrose-containing medium, however, will affect the development of photoautotrophy in coconut vitroplants that can, in turn, affect plant performance when transferred *ex vitro*.

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