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Research note

Developmental changes in carboxylase activities in *in vitro* cultured coconut zygotic embryos: comparison with corresponding activities in seedlings

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Abstract

Phosphoenolpyruvate Carboxylase (PEPC; EC: 4.1.1.31) and Ribulose 1,5-bisphosphate Carboxylase / Oxygenase (RubisCO; EC: 4.1.1.39) enzyme specific activities were measured during the *in vitro* development of coconut (*Cocos nucifera* L.) zygotic mature embryos into plantlets and compared with those of palms produced by conventional seed germination. At the time of initiation of germination, high PEPC and low RubisCO activities were measured in both cultured and conventionally germinated embryos, thus indicating an anaplerotic CO_2 fixation. During both *in vitro* and *in planta* development, RubisCO progressively took over and became the main route for inorganic carbon fixation. The *in vitro*-grown coconut plantlets showed a faster decrease in their PEPC:RubisCO ratio than the seedlings, suggesting that an earlier transition from a heterotrophic to an autotrophic mode of carbon fixation takes place in the *in vitro*-derived material. Just before acclimatization, the RubisCO activity in *in vitro*-derived plantlets (2.83 μmol $CO_2 h^{-1} mg^{-1}{}_{TSP}$) was lower than that in seedlings (6.98 μmol $CO_2 h^{-1} mg^{-1}{}_{TSP}$) of the same age. Nevertheless, after acclimatization, RubisCO activities were comparable in both *in vitro* and *in planta* germinated material

Abbreviations: FW – Fresh Weight; PAR – Photosynthesis Active Radiation; PEPC – Phosphoenolpyruvate Carboxylase (EC: 4.1.1.31); RH – Relative Humidity; RubisCO – Ribulose 1,5-bisphosphate Carboxylase / Oxygenase (EC: 4.1.1.39); T° – Temperature; TSP – Total Soluble Protein

In vitro culture of zygotic embryos provides a means of overcoming, during the collection and exchange of germplasm, the multiple constraints imposed by the heavy weight, large size and lack of dormancy of coconut (*Cocos nucifera* L.) seeds. Conditions for the *in vitro* culture of coconut zygotic embryos have been well documented (Assy-Bah, 1993). Coconut plantlets originating from zygotic embryo cultures have been reported to show a reduced and slower development after acclimatization compared with seedlings (Assy-Bah et al., 1989). Success rates during this final step are generally dependent upon the physiological status of the *in vitro*-grown plantlets and thus, at least partly, upon their photosynthetic ability (Van Huylenbroeck and Debergh, 1996).

The study of the two primary carboxylating enzymes PEPC and RubisCO in *in vitro*-grown plantlets is of paramount interest, since it enables the estimation of the relative importance of non-photosynthetic (heterotrophic) and photosynthetic (autotrophic) carbon fixation (Hdider and Desjardins, 1994; Kumar et al, 1988; Rival et al., 1996, 1997).

The work described here was aimed at monitoring the development of carboxylase activities during the growth of coconut zygotic embryos, comparing the situation in *in vitro* cultured and conventionally germinated material.

All measurements were carried out on seeds and zygotic embryos of the autogamous Malaysian Yellow Dwarf coconut (*Cocos nucifera* L.) ecotype. Embryos were sampled and cultivated *in vitro* according to the protocol of Assy-Bah et al. (1989). Coconut seednuts were cultivated in a computer monitored tropical glasshouse (T°: 27 ± 2 °C, RH: 70 ± 5%, PAR: 180 - 200 μmol m^{-2} s^{-1}).

For enzyme analyses, leaf samples averaging 100 mg FW were harvested at various stages during *in vitro* and *in planta* development. PEPC and RubisCO were extracted from fresh leaves according to Rival et al. (1996). PEPC and RubisCO (Mg^{2+}-activated) activities were assayed following the incorporation of ^{14}C labelled Na bicarbonate into acid stable products in the crude enzyme extracts as previously described (Nato and Mathieu, 1978). All measurements were performed in triplicate on at least 2 different plants. The studied stages are described in Table 1.

The influence and interaction of the culture process and the developmental factors on PEPC and RubisCO specific activities and on the PEPC:RubisCO ratio were analysed using two-way ANOVA. Categorical means were compared with the Newman and Keuls test. Since RubisCO activities were extremely low before stage II (0.18 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$ in seed-derived plantlets and 0.05 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$ in *in vitro*-grown plantlets), any small changes in the activity of this carboxylase at the early stages of development (stages 0 and I) resulted in considerable changes in the PEPC:RubisCO ratio. For this reason, PEPC:RubisCO ratios were statistically analysed only from the stage II.

A two-way ANOVA was performed in order to test the effect of the culturing protocol and culture stage and their interaction on PEPC and RubisCO activities. All effects were significant ($F>7.3$; $p<0.02$), except for the effect of the culturing protocol on RubisCO activity, which was not ($F=3.8$; $p=0.06$). Since all interactions were significant, both factors were used in multiple comparison of means (Newman and Keuls' test).

From stage O to stage I, PEPC specific activity was found to decrease from 17.86 (in both conditions) to 4.54 μmol $CO_2h^{-1}mg^{-1}{}_{TSP}$ *in vitro* and 3.41 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$ *in planta*, whereas no significant change was found in RubisCO activity. After 15 days in culture (stage I), high PEPC activities were measured in both the *in vitro* and *in planta* germinated embryos, thus providing evidence of a preferential carbon fixation through the anaplerotic PEPC pathway (Figure 1). This transient behaviour, observed when C_3-type

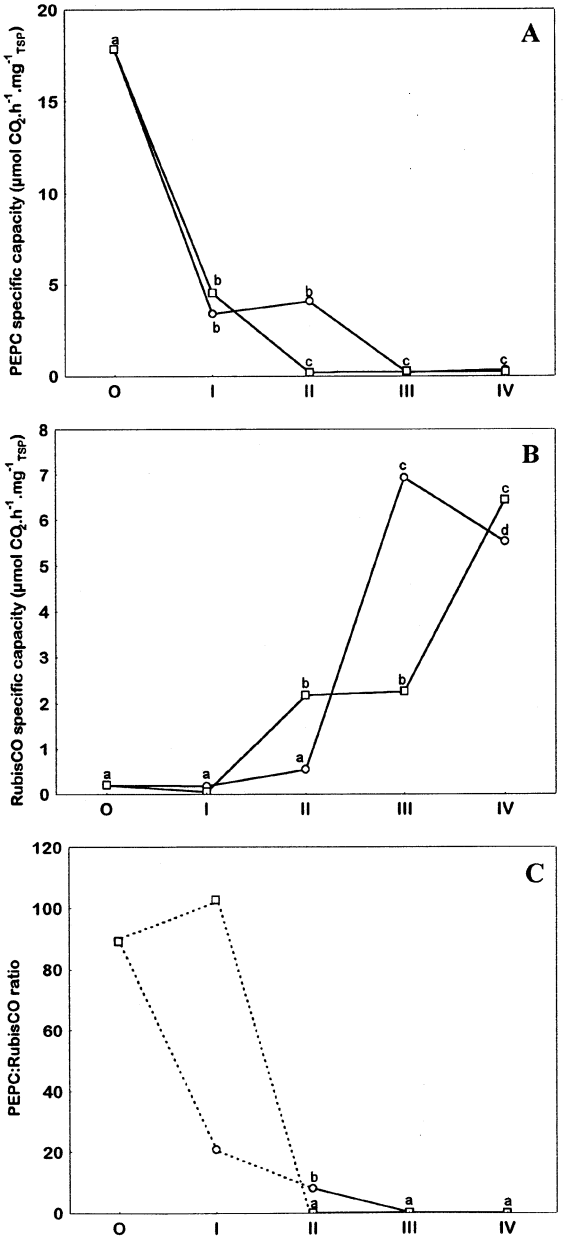

Figure 1. Changes in PEPC (*A*) and RubisCO (*B*) specific activities and PEPC:RubisCO ratios (*C*) during the development of coconut *in vitro* grown plantlets (□) and seed-derived plantlets (◯). For PEPC: RubisCO ratios, the dashed lines represent data not statistically analysed. Values followed by the same letter were not significantly different as determined by the Newman and Keuls' test.

plants or isolated cells are grown *in vitro*, has already been described in various species (Desjardins, 1995; Hdider and Desjardins, 1994; Neuman et al., 1989; Rival et al., 1997). PEPC may furnish the anaplerotic

Table 1. Studied stages of *in vitro* development and seednut germination.

Stage	*In vitro* culture duration (days)	Time after sowing (days)	Culture stage (*in vitro*)	Culture stage (seed germination)
0	0	0	inoculation	sowing
I	15	15	appearance of root and cotyledon	appearance of root and cotyledon
II	105	105	light green leaf (2 to 3 leaves)	light green leaf (2 to 3 leaves)
III	180	195	green leaf (2 to 3 leaves)*	green leaf (5 to 6 leaves)
IV	210	225	after acclimtization	> 6leaves

*plantlet ready-to-acclimatization

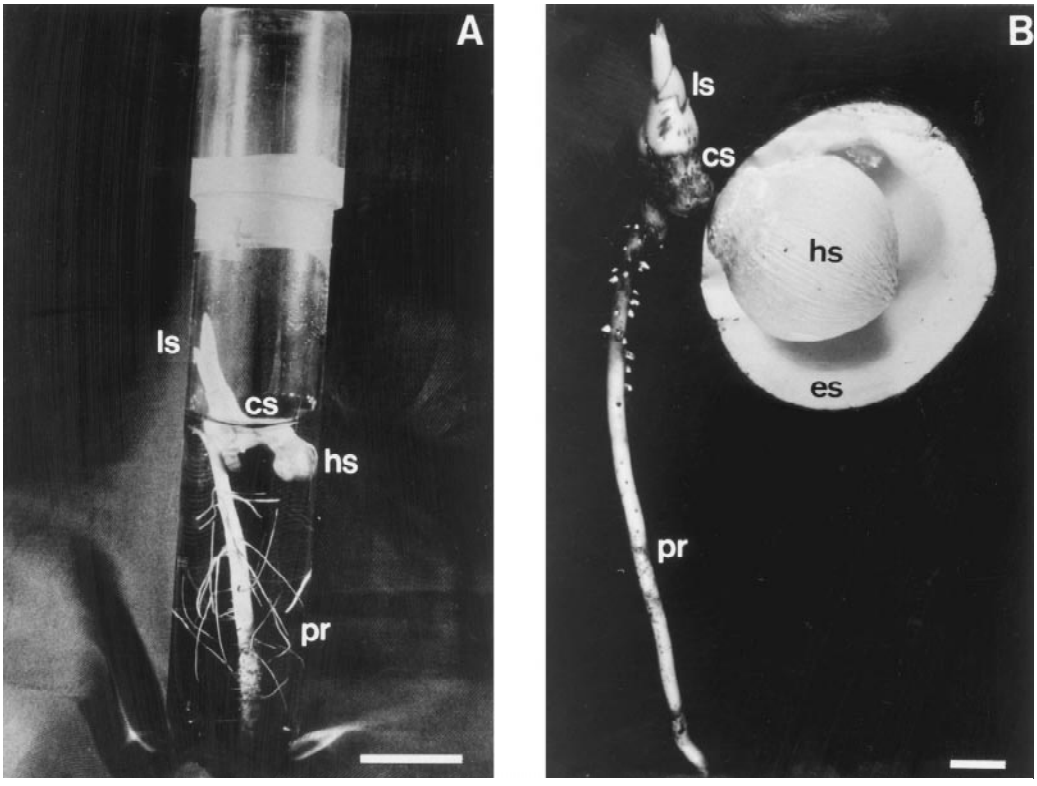

Figure 2. Comparison of coconut *in vitro* grown (*A*) and conventionally germinated plantlets (*B*) from stage I. Scale bar = 2 cm. cs: cotyledonary stalk; es: endosperm; hs: haustorium; lf: leaf scale; pr: primary root.

carbon supply for aminoacid and protein synthesis during the early stages of *in vitro* plantlet culture, when the demand is considerable (Kumar et al., 1988).

At stages I and II, significant differences were observed between plantlets cultivated *in vitro* and seed-derived plantlets with respect to both PEPC and RubisCO carboxylase activities. In seed-derived plantlets, the significant decrease in PEPC and increase in RubisCO activities seen were noted only at stage II, while in *in vitro*-cultured plantlets these changes were observed at stage I. Thus, the PEPC:RubisCO ratio decreased faster in *in vitro*-grown plantlets than in seedlings. The carboxylase ratio in *in vitro*-grown plantlets was significantly lower than in seednut-derived plants (0.09

and 8.15 respectively; see Figure 1). Decrease in the carboxylase ratio has been previously associated with a transition from a heterotrophic to a more autotrophic mode of carbon fixation (Hdider and Desjardins, 1994; Rival et al., 1996b). Thus, it appeared that transition to autotrophic CO_2 fixation was earlier in *in vitro* than in seednut-derived plantlets.

The development was observed to be faster in seedlings, which bore at least 5 leaves after 6 months, as compared with 3 leaves in the case of *in vitro*-grown plantlets. The faster development observed in seedlings might be attributable to the large amount of reserves found in the coconut seednut (at least 1000 times the embryo fresh weight), in contrast to the relatively poor amount of nutrients provided *in vitro* by the culture medium (Jayaleksmy et al., 1988). *In planta*, the digestion and mobilisation of the reserves are carried out by a specific tissue, the haustorium, through the activity of various degradative enzymes (Nagarajan and Panladai, 1965). This tissue was very poorly developed in *in vitro*-grown plantlets. Foale (1968) reported that reserves in the coconut endosperm were totally exhausted when the seedlings were 18 months old, thus suggesting an extremely late attainment of autotrophy in this species.

At stage III, the main difference observed that could be linked to the tissue culture process is the lower RubisCO activity of ready-to-acclimatize *in vitro*-grown plantlets (2.83 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$) compared with that of seedling-derived plants (6.93 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$). This result has been reported for many C_3 species cultivated *in vitro* and the lower RubisCO activity has been mainly attributed to the repressive effect of sucrose (Desjardins, 1995; Van Huylenbroeck and Debergh, 1996). The presence of high levels of sucrose (60 g l^{-1}) throughout the culture process of coconut zygotic embryos (Assy-Bah et al., 1989) might play a role in the decrease of RubisCO activity seen in *in vitro*-grown plantlets (Neumann et al., 1989). After acclimatization, the RubisCO activity increased up to 7.06 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$, thus reaching a value comparable to the activity measured in seedlings (6.15 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$).

The process of *in vitro* embryo culture described by Assy-Bah (1989) and modified by Rival et al. (1996) allows good survival rates, often reaching 95%. *In vitro*-grown plantlets thus appear to successfully withstand the acclimatization step and to achieve further autotrophic growth.

The preliminary study presented here now needs to be complemented with an investigation of *in planta* photosynthetic parameters, such as CO_2 exchange and patterns of chlorophyll fluorescence (Desjardins, 1995; Rival et al., 1996b), together with a precise quantification of carboxylating enzymes through immunoenzymatic techniques (Rival et al., 1996a, b).

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