Plant Cell, Tissue and Organ Culture **57**: 139–143, 1999.

Research note

Carbon metabolism in *in vitro* cultures of date palm: the role of carboxylases (PEPC and RubisCO)

Raja Masmoudi[1], Alain Rival[2]*, Aimé Nato[3], Danièle Lavergne[3], Nourredine Drira[1] & Georges Ducreux[3]

[1]*Laboratoire de Physiologie Végétale, Faculté des Sciences de Sfax, 3038 Sfax, Tunisie;* [2]*Laboratoire GeneTrop. IRD ex ORSTOM/CIRAD-CP. Centre IRD, B.P. 5045, 34032 Montpellier Cedex 01, France;* [3]*Laboratoire de Morphogenèse Végétale Expérimentale, Université Paris Sud, Bât 360, 91405 Orsay Cedex France (*requests for offprints; Fax: +33(0)4 6741 6181; e-mail: rival@ird.fr)*

Received 7 January 1999; accepted in revised form 12 August 1999

Key words: carbon metabolism, carboxylases, CO_2 fixation, *Phoenix dactylifera* L., somatic embryogenesis

Abstract

While describing major trends of carbon metabolism during the initiation and expression of somatic embryogenesis in date palm (*Phoenix dactylifera* L., cv. Deglet Nour), we have investigated the role of two carboxylases, namely PEPC (Phosphoenolpyruvate carboxylase, EC 4.1.1.31) and RubisCO (Ribulose 1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39), in embryogenic and non-embryogenic cultures. The detection of PEPC activity on polyacrylamide native gels after electrophoresis revealed the presence of 3 active isoforms in crude extracts from the embryogenic (E) callus strain, whereas only a single band was present in the non-embryogenic (NE) one. The level of PEPC specific capacity was of the same order (3.9 ± 1.2 μmol CO_2 h^{-1} mg^{-1} TSP) in both types of cultures. Further changes in carboxylase (PEPC and RubisCO) activities during the growth and development of somatic embryo–derived plantlets were also analysed. The PEPC/RubisCO ratio was found to progressively decrease (from 17.7 to 0.2) throughout the *in vitro* development of plantlets, due to a substantial depletion of PEPC activity, which decreased from 5.3 to 1.2 μmol CO_2 h^{-1} mg^{-1} TSP. Concomitantly, RubisCO assumed greater importance (from 0.3 to 5.3 μmol CO_2 h^{-1} mg^{-1}_{TSP}) and became the main route for inorganic carbon fixation. Western blot analysis using polyclonal antibodies raised against PEPC and RubisCO purified from tobacco leaves confirmed this trend in terms of relative enzyme abundance.

Abbreviations: FW – fresh weight; PEPC – phosphoenolpyruvate carboxylase (EC 4.1.1.31); RubisCO – Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); SE – somatic embryogenesis; TSP – total soluble proteins

Date palm (*Phoenix dactylifera* L.) is widely grown in North Africa and the Middle East and it is the basic component of the oasis. *In vitro* micropropagation plays a very important role in breeding strategies for date palm, which mainly focus on resistance to *Bayoud*, caused by *Fusarium oxysporum* Schlechtend. Fr. f.sp. *albedinis* (Fernandez et al., 1995). Several methods for the *in vitro* micropropagation of date palm have been reported. *In vitro* organogenesis by the reversion of floral buds was first described (Drira and Benbadis, 1985). Somatic embryogenesis, which has been successful for other palm species (Pannetier et al., 1981; Buffard-Morel et al., 1992), now appears to be a very promising approach for the large scale propagation of date palm (Daguin and Letouze, 1988; El Hadrami et al., 1995). The origin and nature of embryogenic cultures is of critical importance for the *in vitro* production of somatic embryos (Veramendi and Navarro, 1997). Indeed, these authors reported that only the nodular callus originating from leaf primordia bases could produce viable somatic embryos.

Attention in our group has focused on the study of carbon metabolism during somatic embryogenesis of date palm. In order to point out putative differences in biochemical activity which may differentiate embryogenic from non-embryogenic callus cultures at the early stages of the SE process, phosphoenolpyruvate carboxylase (PEPC, EC: 4.1.1.31) patterns were investigated. PEPC is a multi-faceted enzyme (Chollet et al., 1996) which has been described as very active in *in vitro* cultured plant cells (Lavergne et al., 1992; Kwa et al., 1997; Rival et al., 1997; Triques et al., 1997).

The date palm, like many *in vitro* grown C_3 plants including oil palm and coconut palm (Rival et al., 1997; Triques et al., 1997), can assimilate inorganic carbon via two independent carboxylation pathways. One occurs in the chloroplasts through the action of RubisCO. The activity of this enzyme is closely linked to the development of chloroplasts and reflects the integrity of the photosynthetic apparatus. The other pathway takes place in the cytosol through the Phosphoenolpyruvate carboxylase pathway. The ratio of PEPC:RubisCO activities is considered to be a reliable indicator of the relative conditions of non-photosynthetic (heterotrophic) and photosynthetic (autotrophic) pathways of CO_2 fixation (Kumar et al., 1988; Kwa et al., 1997).

The objectives of the work described in the present article are the following: (i) to study comparatively the activity and accumulation of PEPC isoforms in embryogenic and non-embryogenic callus cultures of date palm and (ii) to monitor the changes occurring in the PEPC:RubisCO ratio during the *in vitro* development of somatic embryos into plantlets.

Embryogenic cultures (E) used in the present study were derived from axillary and apical vegetative buds originating from offshoots of an adult date palm (*Phoenix dactylifera* L., cv. Deglet Nour). Non-embryogenic (NE) cultures were isolated using leaf explants from the same genotype. Throughout the SE protocol of date palm, the culture medium of Murashige and Skoog (1962) was adapted as follows: sucrose (50 g l^{-1}), myo-inositol (100 mg l^{-1}), glycine (2 mg l^{-1}), L-glutamine (100 mg l^{-1}), KH_2PO_4 (120 mg l^{-1}) and adenine (25 mg l^{-1}) were added. For culture initiation/induction, 2,4-D was used at 5 mg l^{-1} concentration. Primary explants were cultivated for 4 to 5 months in darkness at 27 °C on the 2,4-D-enriched medium. Cultures were then cultivated in light under a 16-h photoperiod and transferred every 5 weeks onto hormone-free medium.

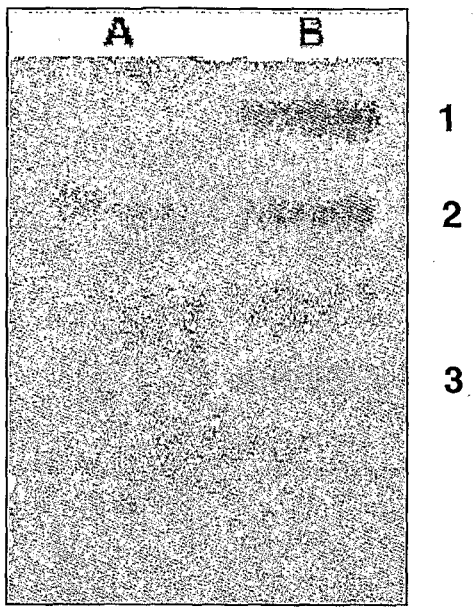


Figure 1. Fast Violet Blue detection of PEPC active isoforms on polyacrylamide native gel after the electrophoretic separation of total soluble proteins (30 μg TSP per sample). Lane A: Non-embryogenic callus cultures; Lane B: Embryogenic callus cultures.

Histological examinations of E and NE cultures were performed at various growth stages using the protocol of Buffard-Morel et al. (1992). NE cultures displayed only somatic cells with the absence of characteristic meristematic nodules or very young pre-embryos.

Extraction and quantification of total soluble proteins (TSP), PEPC and RubisCO activities were performed as previously described (Rival et al., 1997). Western blots were carried out according to Lavergne et al. (1992) using polyclonal antibodies raised against carboxylases purified from tobacco leaves. Cross-reactivity between anti-PEPC polyclonal antibodies from tobacco and various palm species (Rival et al., 1997) has been previously verified using Ouchterlony double-diffusion tests, thus confirming the similarity of PEPC structure amongst C3 plants (Matsuoka and Hata, 1987). PEPC activity was revealed on native polyacrylamide gels using the Fast Violet Blue reaction (Vidal et al., 1976). TSP content was much higher (2.11 vs. 1.37 $mg_{prot}\ g^{-1}{}_{FW}$) in type E cultures than in NE ones, suggesting that metabolic activity is more intense. Specific PEPC activity was determined from crude extracts: the level of activity was found to be of the same order (3.9 $\pm$ 1.2 μmol CO_2 h^{-1} mg^{-1}prot.) in both types of cultures. Nevertheless, the

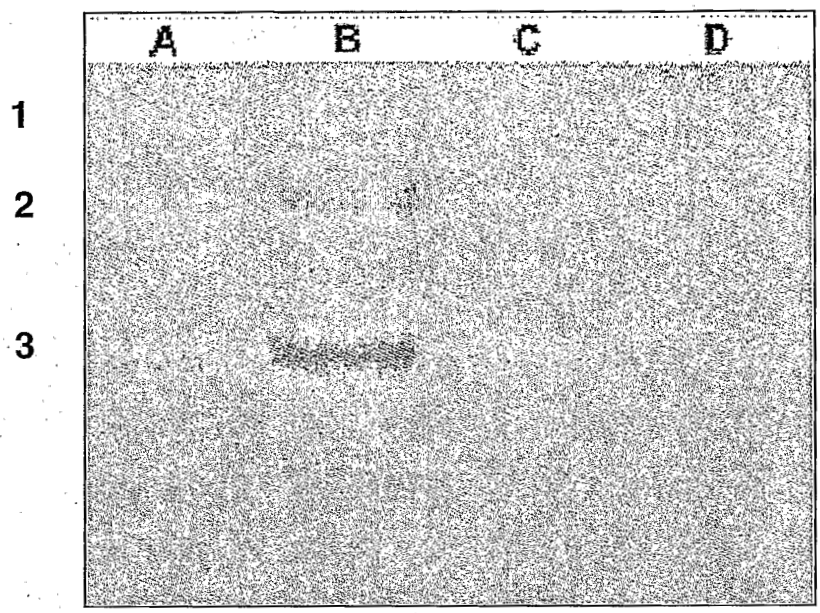


Figure 2. Fast Violet Blue detection of PEPC active isoforms on polyacrylamide native gel after the electrophoretic separation of total soluble proteins (30 μg TSP per sample). Lane A: young somatic embryos; Lane B: maturated somatic embryos; Lane C: plantlets (1 leaf); Lane D: plantlets (2–3 leaves).

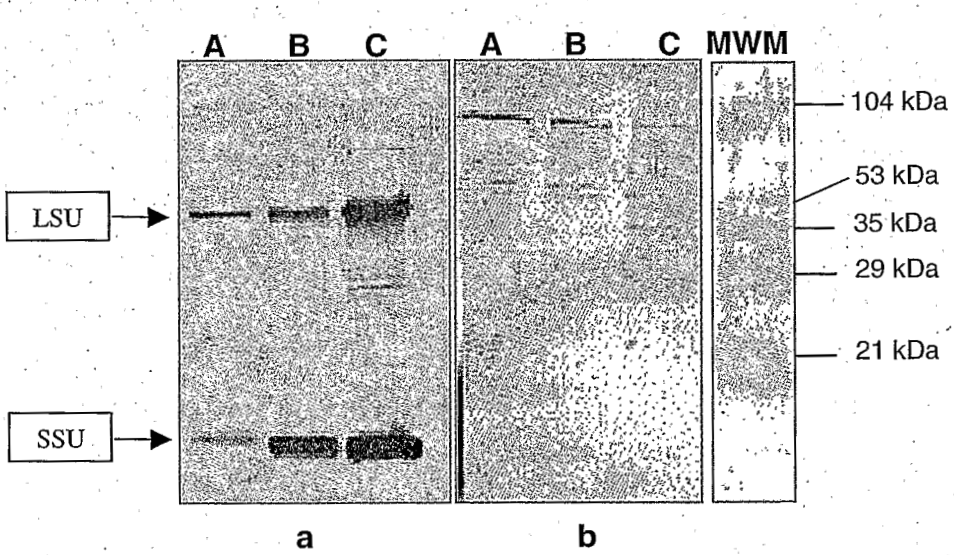


Figure 3. Western blot analysis after SDS-PAGE of RubisCO (a) and PEPC (b) carboxylases from date palm crude extracts sampled at various stages of somatic embryos development. Lane A: young somatic embryos; Lane B: plantlets (1 leaf); Lane C: plantlets (2–3 leaves). MWM: Molecular weight markers. TSP amounts in wells were 1 μg for RubisCO detection and 30 μg for PEPC detection.

detection of PEPC activity after native electrophoresis on polyacrylamide gels revealed the expression of 3 active isoforms (1, 2 and 3) of the enzyme in crude extracts originating from E calli, whereas only one unique isoform (2) was detected in NE cultures (Figure 1). Furthermore, immunoblot analyses performed after SDS-PAGE revealed a single band of 100 kDa in both extracts (results not shown). Thus, it can be assumed that only the 100 kDa isoform contains the antigenic domain detected by the polyclonal antibody (anti-PEPC from tobacco leaves) used.

Specific PEPC and RubisCO activities were estimated on the same crude extracts originating from somatic embryos and plantlets sampled at various de-

Table 1. Changes in carboxylase activities during the *in vitro* development of date palm somatic embryos. Each value is the mean of at least 3 different measurements in triplicate

Culture stage	TSP (mg g_{FW}^{-1})	PEPC specific activity*	RubisCO specific activity*	PEPC:RubisCO ratio
Proliferating embryos	2.0±0.3	5.3±0.7	0.3±0.1	17.66
Plantlets (1 leaf)	2.7±0.6	0.9±0.3	1.2±0.2	0.75
Plantlets (2–3 leaves)	2.5±0.5	1.2±0.4	5.3±0.7	0.22

*μmol $CO_2.h^{-1}.mg^{-1}{}_{TSP}$.

velopmental stages (Table 1). The PEPC:RubisCO ratio constantly decreased throughout the development of somatic embryos, thus suggesting a progressive transition towards a RubisCO-mediated photosynthetic CO_2 incorporation. Inorganic carbon fixation was carried out mostly by PEPC in proliferating embryos, reflecting a heterotrophic carbon metabolism (ratio = 17.66). These results, if compared to those obtained with somatic embryos of oil palm (*Elaeis guineensis* Jacq) by Rival et al. (1997), show that PEPC activity is at a comparable level in both materials sampled at the same stage, but that RubisCO specific activity is much higher in oil palm (3.37 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$), thus leading to a lower PEPC:RubisCO ratio (1.42).

The decrease in PEPC specific activity was found to be concomitant with the progressive disappearance of isoforms 1 and 2, thus leading to the presence of only isoform 3 in plantlets at the 2–3 leaf stage (Figure 2). Furthermore, western blot analysis of RubisCO revealed that the relative amount of both LSU and SSU sub-units increased continuously throughout the *in vitro* development of somatic embryos (Figure 3a). These changes were concomitant with a significant increase in the specific capacity of the enzyme, from 0.3 to 5.3 μmol CO_2 h^{-1} $mg^{-1}{}_{TSP}$ (Table 1). The decrease in PEPC specific capacity (Table 1) corresponds to a decrease in PEPC relative abundance, as shown in Figure 3b.

The analysis of PEPC enzyme isoforms has revealed that embryogenic cultures may be characterised through specific patterns. This result must now be confirmed using several other date palm genotypes and *in vitro* culture conditions before it can be used as a predictive marker for the competence of callus cultures to become embryogenic. The presence of several active isoforms of PEPC in plant organs (Gehrig et al., 1998) supports the view that housekeeping isoforms coexist with other isoforms related to more specific functions. Each member of the PEPC multigenic family is located on a specific chromosome and encodes a distinct isoform which is associated with its respective metabolic pathway (Dong et al., 1998). In our case, specific PEPC isoforms detected in E cultures are probably involved in functions linked with the early stages of somatic embryogenesis, such as the anaplerotic supply of carbon skeletons to actively proliferating and differentiating cells or the control of intracellular osmolarity (Latzko and Kelly, 1983). In order to clarify the role of the PEPC isoforms detected in E cultures, the presence of these polypeptides will be also investigated in young somatic embryos.

Our results also demonstrate the establishment of a photosynthetic type metabolism during the *in vitro* development of date palm somatic embryos. Several notable similarities have been observed in patterns of developmental changes in carboxylases between date palm *in vitro*-grown plantlets on one hand and coconut (Triques et al., 1997) and oil palm (Rival et al., 1997) plants on the other.

This work now needs to be complemented with an investigation of *in planta* photosynthetic parameters, such as CO_2 exchanges and patterns of chlorophyll fluorescence (Rival et al., 1997), together with a precise quantification of carboxylating enzymes through immuno-enzymatic techniques (Rival et al., 1996).

Acknowledgements

Thanks are due to Dr J. Tregear for English corrections. Dr K. Triques is gratefully acknowledged for fruitful and stimulating discussions.

References

Buffard-Morel J, Verdeil JL & Pannetier C (1992) Embryogenèse somatique du *cocotier (Cocos nucifera* L.) à partir de tissus foliaires: Etude histologique. Can. J. Bot. 70: 735–741

Chollet R, Vidal J & O'Leary MH (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol Biol. 47: 273–298

Daguin F & Letouze R (1988) Régénération du palmier dattier (*Phoenix dactylifera* L.) par embryogenèse somatique: amélioration de l'efficacité par passage en milieu liquide agité. Fruits 43: 191–194

Drira N & Benbadis A (1985) Multiplication végétative du palmier dattier (*Phoenix dactylifera* L.) par reversion en culture *in vitro* d'ébauche florale de pieds femelle. J. Plant Physiol. 119: 227–235

El Hadrami I, Cheik R & Baaziz M (1995) Somatic embryogenesis and plant regeneration from shoot-tip explants in *Phoenix dactylifera* L. Biol. Plant. 37: 205–211

Fernandez D, Lourd M, Ouiten M, Tantaoui A & Geiger JP (1995) The bayoud, a desease threatening date palm. Phytoma 469: 36–39

Gehrig H, Faist K & Kluge M (1998) Identification of PEPC isoforms in leaf, stem and roots of the obligate CAM plant *Vanilla planifolia* Salib (Orchidaceae): a physiological and molecular approach. Plant Mol. Biol. 38: 1215–1223

Kumar PP, Bender L & Thorpe T (1988) Activities of Ribulose bisphosphate carboxylase and phosphoenolpyruvate carboxylase and ^{14}C-bicarbonate fixation during *in vitro* culture of *Pinus radiata* cotyledons. Plant Physiol. 87: 675–679

Kwa SH, Wee YC & Kumar PP (1997) Ribulose-1,5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase activities of photoautotrophic callus of *Platycerium coronarium* (Koenig ex OF Muell.) Desv. under CO2 enrichment. Plant Cell Tiss. Org. Cult. 50: 75–82

Latzko E & Kelly GJ (1983) Many faced function of phosphoenolpyruvate carboxylase in C3 plants. Physiol. Vég. 21: 805–815

Lavergne D, Nato A, Dupuis JM, Péan M & Chagvardieff P (1992) Evidence for the expression of morphological and biochemical characteristics of C_3-photosynthesis in chlorophyllous callus of *Zea mays*. Physiol. Plant 84: 292–300

Matsuoka M & Hata S (1987) Comparative studies of phosphoenolpyruvate carboxylase from C3 and C4 plants. Plant Physiol. 85: 947–951

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473–479

Nato A, Hoarau J, Brangeon J, Hirel B & Suzuki A (1985) Regulation of carbon and nitrogen assimilation pathways in tobacco cell suspension cultures in relation with ultrastructural and biochemical development of the photosynthetic apparatus. In: Neumann KH, Barz W & Reinhard E (eds) Primary and Secondary Metabolism of Plant Cell Cultures (pp 43–57). Springer-Verlag, Berlin

Pannetier C, Arthuis P & Lievoux D (1981) Néoformation de jeunes plantes d'*Elaeis guineensis* à partir de cals primaires obtenus sur fragments foliaires cultivés *in vitro*. Oléagineux 36: 119–122

Rival A, Beulé T, Lavergne D, Nato A, Havaux M & Puard M (1997) Development of photosynthetic characteristics in oil palm during *in vitro* micropropagation. J. Plant Physiol. 150: 520–527

Rival A, Beulé T, Nato A & Lavergne D (1996) Immunoenzymatic study of RubisCO in oil palm and coconut. Plant. Res. Dev. 3: 58–65

Triques K, Rival A, Beulé T, Puard M, Roy J, Nato A, Lavergne D, Havaux M, Verdeil JL, Sangare A & Hamon S (1997) Photosynthetic ability of *in vitro* grown coconut (*Cocos nucifera* L.) plantlets derived from zygotic embryos. Plant Science 127: 39–51

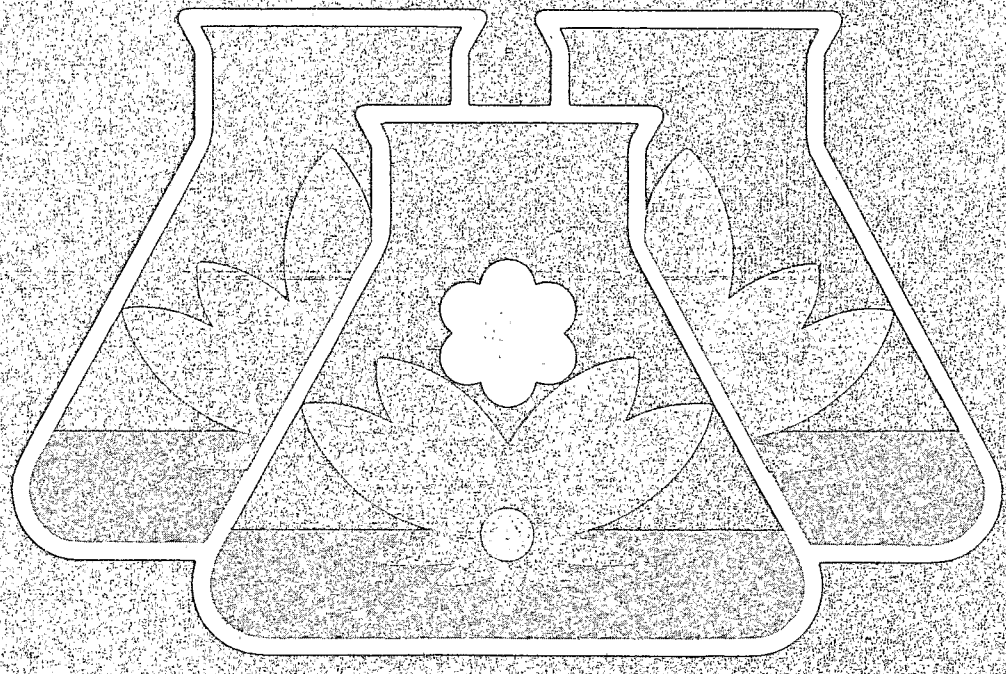
Veramendi J & Navarro L (1997) Influence of explant sources of adult date palm (*Phoenix dactylifera* L.) on embryogenic callus formation. J. Hort. Sci. 72: 665–671

Vidal J, Cavalier G & Gadal P (1976) Etude de la PEPC du haricot et du sorgho par électrophorèse sur gel de polyacrylamide. Plant Sci. Lett. 7: 265–270

Volume 57 No. 2 1999 CODEN PTCEDJ ISSN 0167-6857

Plant Cell, Tissue and Organ Culture

An International Journal on the Cell Biology of Higher Plants



Kluwer Academic Publishers