ELSEVIER

Plant Science 111 (1995) 229–236

PLANT SCIENCE

Specific nutrient uptake during initiation of somatic embryogenesis in coconut calluses

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Received 27 June 1995; revision received 7 September 1995; accepted 11 September 1995

Abstract

Changes in the concentration of major nutrients and sugars in the media were measured for two embryogenic strains of coconut calluses, L1 and L7, after 0, 14, 28, 42 and 56 days of culturing on multiplication medium and embryogenesis induction medium. A histological study was made on day 0, 28 and 56. The initiation of somatic embryogenesis was achieved in these two strains through two different pathways: one (strain L1), of pluricellular origin, was obtained by decreasing the 2,4-D concentration in the medium, and the other (strain L7), of unicellular origin, by increasing the level of 2,4-D. In spite of these differences, both kinds of embryogenesis initiation were linked to specific requirements: higher uptake per g of dry matter of NH_4^+, Ca^{2+}, Mg^{2+} and sucrose.

Keywords: *Cocos nucifera*; 2,4-D; Mineral nutrients; Sugar; Embryogenic calluses

1. Introduction

In a previous study [1], in vitro growth of two strains of coconut calluses and changes in the concentration of the major nutrients and sugars in the medium were analyzed. Both strains studied, L1 and L7, could be distinguished by two main aspects: their optimal growth was controlled by two different concentrations of 2,4-D and the organization of their meristematic area was very different.

The initiation of somatic embryogenesis is achieved in these two strains in two different ways as previously demonstrated [2–4]: strain L1, of pluricellular origin, is obtained by decreasing the 2,4-D concentration in the medium, and strain L7, of unicellular origin, by increasing the level of 2,4-D.

Mineral and carbohydrate requirements for the initiation of somatic embryogenesis in vitro have been established for numerous plant species [5]. Very often, some parameters of the culture medium, more particularly the carbohydrate and nitrogen sources, have been defined empirically in a very precise way to obtain optimal results. It ap-

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog.

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0168-9452/95/$09.50
SSDI 0168-9452(95)04248-S

pears that sucrose is the most effective carbohydrate source for somatic embryogenesis in the species examined so far [5] and that NH_4^+ is strictly required for the initiation of embryogenesis in *Daucus carota* [6,7], *Atropa belladona* [8], *Solanum melongena* [9] and *Medicago sativa* [10]. To our knowledge, a specific requirement for the other nutrients has never been mentioned, except for K^+, which has been shown to be essential in *Daucus carota* embryogenesis [11].

In most studies, specific requirements have been set out by varying the concentration of the nutrients in the medium and by measuring the effects of these variations on the studied explant. In our study, we measured the changes in the concentrations of all the major nutrients and sugars in the medium. Comparing the nutrient uptake of calluses cultured on multiplication medium and those of calluses cultured on a medium for embryogenesis initiation provides evidence showing specific requirements during the initiation of embryogenesis in *Cocos nucifera.*

2. Materials and methods

Plant material, media preparation, general culture conditions, macroelements and sugars measurements have been described in previous studies [1,12]. The media contained modified MS major elements: KNO_3, 1200 mg/l; KH_2PO_4, 700 mg/l; NH_4NO_3, 1300 mg/l; $CaCl_2.2H_2O$, 360 mg/l; $MgSO_4.7H_2O$, 300 mg/l; MS minor elements [13], Morel and Wetmore vitamins [14], 40 mg/l sulphate adenine, 30 g/l sucrose, 2 g/l activated charcoal and 7.5 g/l agar. Multiplication media, M2 for strain L7 and M3 for strain L1, contained 2.7×10^{-4} M and 3.6×10^{-4} M 2,4-D, respectively. Media for embryogenesis initiation, M1 for strain L1 and M5 for strain L7, contained 1.8×10^{-4} M and 5×10^{-4} M 2,4-D, respectively. Calluses of strain L1 were cultured on M1, M3 and M5 media and those of strain L7 on M1, M2 and M5 media. Measurements were made on days 0, 14, 28, 42 and 56. Each set of conditions (strain × day × 2,4-D concentration) was represented by 4 samples.

Major element concentrations were analyzed by ionic HPLC (Dionex 4500i, Dionex Corporation, Sunnyvale, USA). Anion concentrations were measured using an isocratic method (eluent: 3.9 mM $NaHCO_3$, 3.1 mM Na_2CO_3; guard column IONPAC AGSA; analytic column IONPAC ASSA). For cations, a step-gradient was applied by changing the eluent 3 min after injection (eluent 1: 12 mM HCl and 0.5 mM 2,3-diaminoproprionic acid monohydrochloride; eluent 2: 48 mM HCl and 8 mM 2,3-diaminoproprionic acid monohydrochloride; guard column IONPAC CG3; analytic column IONPAC CS3).

Glucose, fructose and sucrose concentrations were measured with the Bergmeyer and Bernt [15] enzymatic method.

For the measurement of total protein content, calluses were crushed in liquid nitrogen and homogenized in 5 ml of extraction buffer for 10 min, then resuspended and centrifuged for 20 min at 2000 × *g* and at 4°C. The extraction buffer consisted of 50 mM Tris–HCl (pH 7.2), 10 mM NaCl, 1 mM $CaCl_2$, 1 mM dithiothreitol and 0.2% Triton-X100. The proteins in the supernatant were analysed according to Bradford's method [16]. Three measurements were done for each sample.

Histological observations were carried out on days 0, 28 and 56 acccording to the method described by Buffard-Morel et al. [2].

2.1. Statistical analysis

On days 14, 28, 42 and 56, uptake of major nutrients and sugars per g of dry matter of calluses was calculated as follows:

$$\text{Uptake} = \frac{A_i - A}{DW - DW_i}$$

where A is the residual amount of a nutrient in the medium, A_i the initial one (day 0), DW is the dry weight of the callus and DW_i is the dry weight on day 0.

A two-way analysis-of-variance was applied to test the effects of time, 2,4-D concentration, and the time-2,4-D concentration interaction on dry weight of calluses, changes in the uptake of major nutrients and sugars per g of dry matter by calluses and total protein content in the calluses. Time and 2,4-D concentration were fixed-effect factors. When an effect was significant, the Newman and Keuls' test was used for multiple comparison of

categorial means [17,18]. On the graphs, at each time, points within brackets are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test.

3. Results

3.1. Histological study

The growth of the calluses on multiplication medium was ensured, for both strains, by peripheral meristematic zones, but their histological structures were different. For strain L1, the meristematic zone was organized in a cambium-like structure, whereas, for strain L7, it corresponded to 4 or 5 non-organized layers of homogeneous cells. In the internal area of the L7 calluses, cells were differentiated into parenchymatous tissue. In the internal zone of L1 calluses several tracheids were present.

In strain L1, the transfer to M5 medium (5×10^{-4} M 2,4-D level) induced the differentiation of the meristematic zone and much of the internal area into vascular elements (Fig. 1a). In strain L7, when transferring on M5 medium, embryogenic

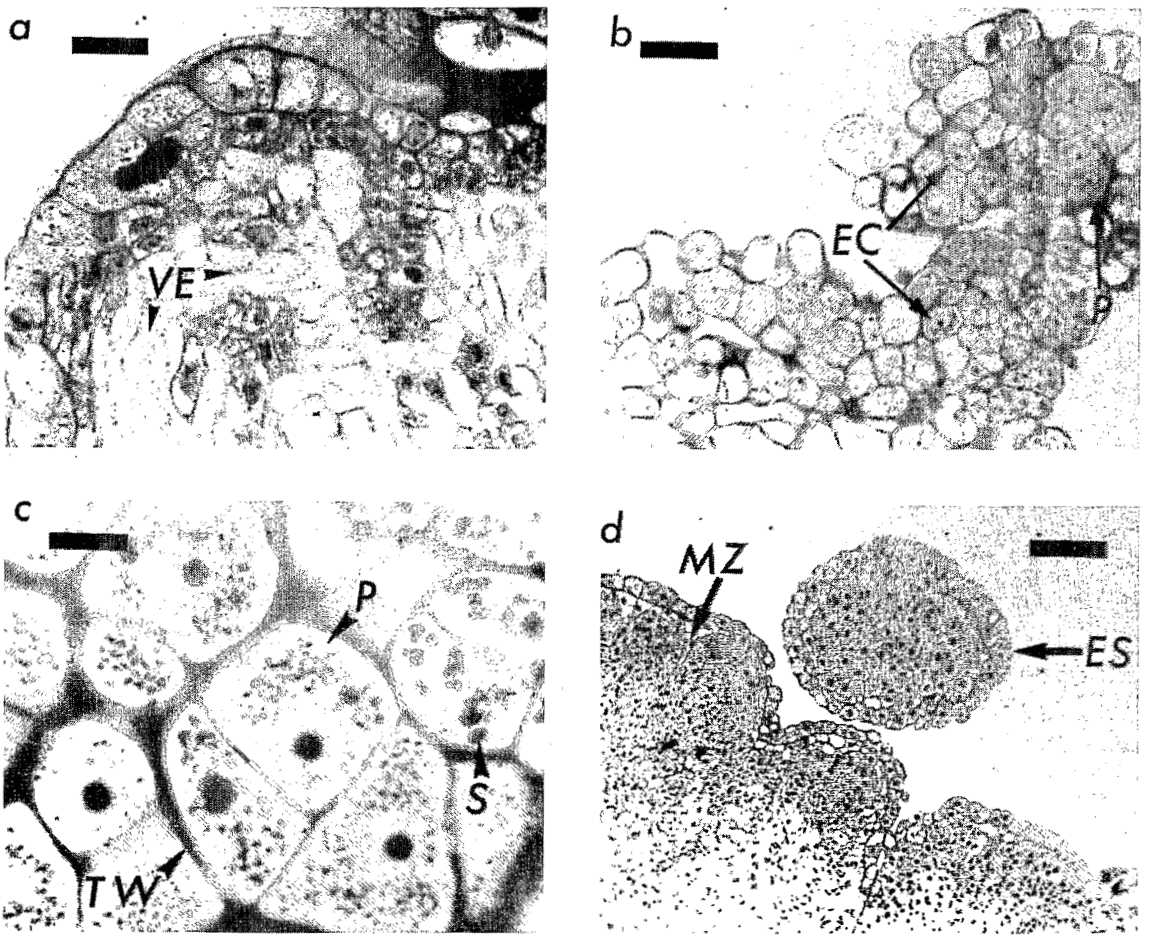

Fig. 1. Strain L1 on M5 medium (a): differentiation of the meristematic zone in vascular elements (VE) (bar, 31 μm). Strain L7 on M5 medium (b): isolation and differentiation of embryogenic cells (EC) and proembryo (P) (bar, 31 μm). Strain L7 on M5 medium (c): proembryos with protein (P) and starch (S) reserves surrounded by a thickened outer wall (TW) (bar, 12 μm). Strain L1 on M1 medium (d): fragmentation of the meristematic zone (MZ) and generation of external embryogenic structures (ES) (bar, 125 μm).

Table 1
Results of two-way analysis-of-variance for strain L1

	2,4-D concentration effect F	Time effect F	2,4-D concentration-time effect F
Dry weight	4.322 *	15.53 ***	0.793 NS
Protein content	6.125 *	8.156 **	0.079 NS
Sucrose uptake	4.561 *	4.346 *	0.591 NS
Glucose uptake	0.326 NS	5.632 *	1.002 NS
Fructose uptake	0.569 NS	7.875 **	0.463 NS
Cl^- uptake	0.239 NS	3.246 *	0.452 NS
$H_2PO_4^-$ uptake	0.819 NS	3.345 *	0.938 NS
NO_3^- uptake	0.975 NS	3.917 *	0.784 NS
SO_4^{2-} uptake	3.068 NS	7.643 **	1.487 NS
Ca^{2+} uptake	5.120 **	6.416 **	0.055 NS
K^+ uptake	2.854 NS	1.549 NS	0.738 NS
Mg^{2+} uptake	4.832 *	6.213 **	0.651 NS
NH_4^+ uptake	10.658 **	3.567 *	0.921 NS

For each effect tested (rows) and trait studied (columns), F value is given.
NS, not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

cells became isolated from the peripheral meristematic zone and developed into proembryos (Fig. 1b). These cells displayed numerous histological characteristics typical of embryogenic cells, as described by Verdeil et al. [3]: a thickened wall, a large centrally positioned nucleus with a densely stained unique nucleolus, a dense cytoplasm with numerous starch and protein reserves (Fig. 1c). Thus, in strain L7, the transfer from 2.7×10^{-4} M to 5×10^{-4} M 2,4-D resulted in the initiation of somatic embryogenesis from a unicellular origin.

In strain L1, the transfer to M1 medium (1.8×10^{-4} M 2,4-D level), led to a fragmentation of the cambium-like meristematic zone which generated external globular structures (Fig. 1d). These glo-

Table 2
Results of two-way analysis-of-variance for strain L7

	2,4-D concentration effect F	Time effect F	2,4-D concentration-time effect F
Dry weight	17.282 ***	45.106 ***	1.046 NS
Protein content	12.227 **	8.913 **	0.546 NS
Sucrose uptake	13.121 **	8.718 **	0.455 NS
Glucose uptake	0.808 NS	7.675 **	0.985 NS
Fructose uptake	1.154 NS	13.425 **	1.065 NS
Cl^- uptake	0.165 NS	9.151 **	0.241 NS
$H_2PO_4^-$ uptake	0.004 NS	22.738 ***	0.080 NS
NO_3^- uptake	0.704 NS	17.125 ***	0.074 NS
SO_4^{2-} uptake	0.449 NS	45.419 ***	1.116 NS
Ca^{2+} uptake	4.125 *	7.613 **	0.680 NS
K^+ uptake	0.015 NS	23.689 ***	0.322 NS
Mg^{2+} uptake	4.325 *	3.537 *	1.035 NS
NH_4^+ uptake	14.903 **	131.740 ***	21.861 ***

For each effect tested (rows) and trait studied (columns), F value is given.
NS, not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

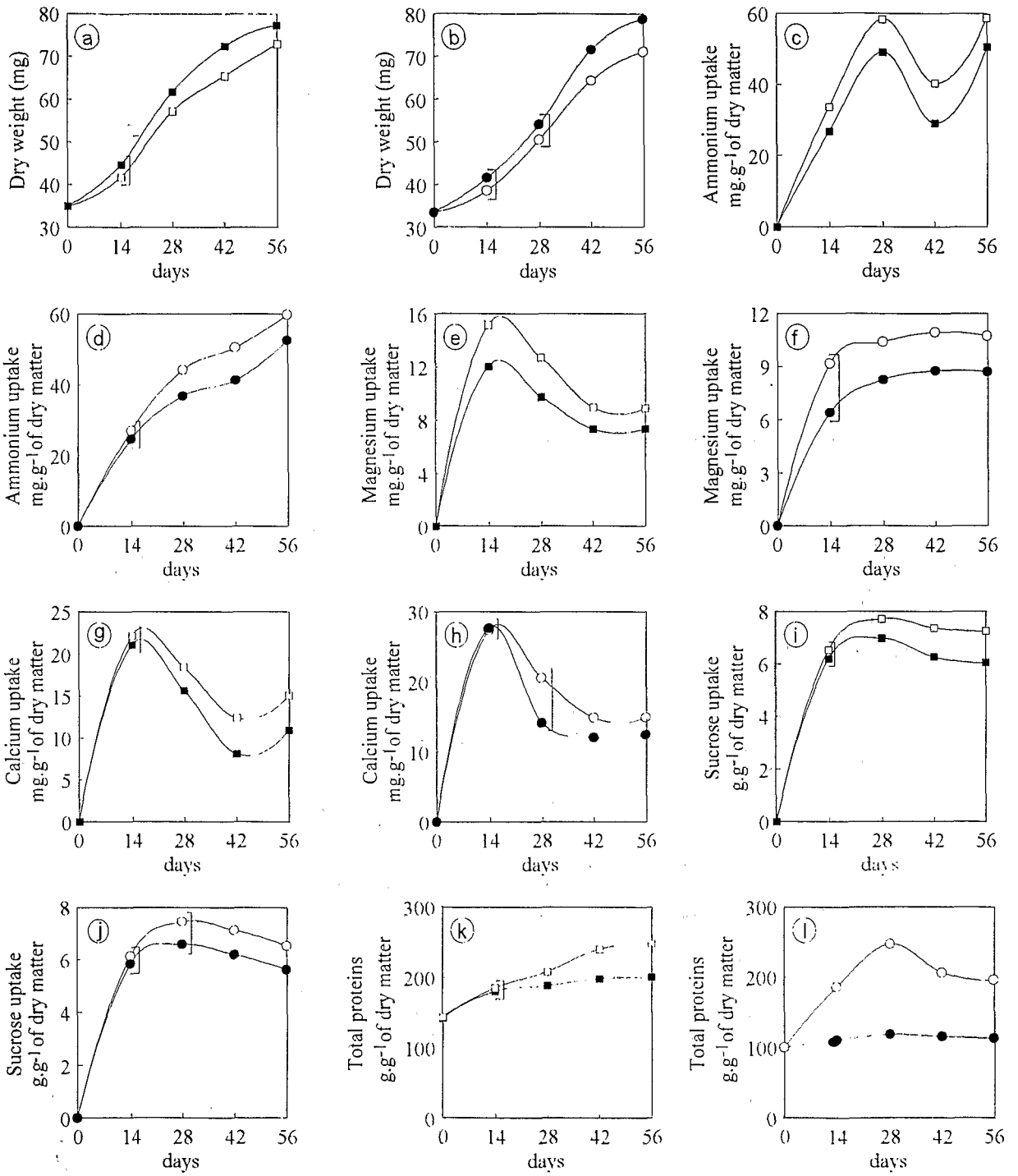

Fig. 2. Changes in the dry weight of calluses (a, b), the uptake of NH_4^+ (c, d), Mg^{2+} (e, f), Ca^{2+} (g, h) and sucrose (i, j) per g of dry matter and the total protein content per g of dry matter (k, l) of the strain L1 (a, c, e, g, i, k), on embryogenesis initiation medium (□) (M1) and multiplication medium (■) (M3), and of the strain L7 (b, d, f, h, j, l), on embryogenesis initiation medium (○) (M5) and multiplication medium (●) (M2). At each time, points within brackets are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test.

bules, which corresponded to embryogenic structures, were composed of highly meristematic cells containing more soluble proteins than the cells cultured on the multiplication medium. These embryogenic structures were very similar to those observed in oil palm [19] and could be oriented to embryo-like forms on a maturation medium [2]. Therefore, in strain L1, the initiation of somatic embryogenesis, from a muticellular origin, was achieved by decreasing the 2,4-D level in the medium. In strain L7, the transfer to 1.8×10^{-4} M 2,4-D did not lead to a noticeable histological change at the end of one subculture.

3.2. Statistics

For all the traits studied and both strains, L1 and L7, two-way ANOVA showed that there was a significant 2,4-D concentration effect for dry weight of calluses, protein content, sucrose, Ca^{2+}, Mg^{2+} and NH_4^+ uptakes (Tables 1 and 2). There was always a time effect, except for K^+ uptake for strain L1.

3.3. Growth

For both calluses strains, transferring to the medium for embryogenesis initiation resulted in a significant decrease of growth (dry weight) as compared to the growth on multiplication medium (3.6×10^{-4} M 2,4-D for strain L1 and 2.7×10^{-4} M 2,4-D for strain L7) (Fig. 2a and 2b).

3.4. Nutrients

The NH_4^+ and Mg^{2+} uptake per g of dry matter were significantly higher on embryogenesis initiation medium compared to those on multiplication medium, starting at day 14 for strain L1 and day 28 for strain L7 (Fig. 2c–f). The sucrose and Ca^{2+} uptake per g of dry matter was also significantly higher, starting at day 28 for strain L1 and day 42 for strain L7 (Fig. 2g–j). Therefore, for both strains, the initiation of somatic embryogenesis was linked to a specific uptake of sucrose, NH_4^+, Ca^{2+} and Mg^{2+}. For all the other nutrients (NO_3^-, SO_4^{2-}, $H_2PO_4^-$, Cl^- and K^+), there was no difference in the uptake per g of dry matter between calluses cultured on the multiplication medium and those cultured on embryogenesis initiation medium. Also, there was no specific uptake for L1 calluses cultured on M5 medium and for L7 calluses cultured on M1 medium.

3.5. Total protein content

The total protein content per g of dry matter was significantly higher on embryogenesis initiation medium compared to that on multiplication medium, starting at day 28 for strain L1 and day 14 for strain L7 (Fig. 2k and l). Thus, for both strains, the initiation of somatic embryogenesis corresponded to a higher protein synthesis.

4. Discussion

For both strains, there was an increase of the total protein concentration in the calluses during the initiation of somatic embryogenesis. Further, histological study showed a high soluble protein content in the cytoplasm for strain L1 and numerous protein reserves for strain L7. This confirms the increase of the protein concentration observed in tobacco [20] and carrot [21,22] calluses during embryogenesis initiation.

This study established the linkage between the two ways of embryogenesis initiation in coconut calluses and specific requirement for sucrose. This requirement can be connected to several phenomena. First, any form of morphogenesis is an energy-demanding process [23]. In addition, the accumulation of starch and the activation of the protein synthesis imply an additional requirement for carbohydrate. With strain L7, the thickening of the cell wall should be equally connected to biosynthesis of polysaccharides generating a greater requirement for carbohydrate.

The initiation of somatic embryogenesis has generated, in L1 and L7, a specific requirement for NH_4^+, while there was no difference in NO_3^- uptake. There was a preferential uptake of the reduced form of inorganic nitrogen for the initiation of somatic embryogenesis. It is established that the presence of reduced nitrogen in the culture medium is favourable to somatic embryogenesis [5,23]. In *Daucus carota*, reduced nitrogen is indispensable to the initation and beneficial to the maturation of somatic embryos [6,7]. In *Atropa belladona* [8], *Solanum melongena* [9] and *Medicago sativa* [10], there is no initiation without

NH_4^+. This preferential requirement for NH_4^+ can be attributed to the increase of protein synthesis during the initiation of somatic embryogenesis. Indeed, it has been established that *Rosa* cells accumulate twice more protein reserves on a medium containing both NH_4^+ and NO_3^- than on a medium containing only NO_3^- [24]. In rice suspension cultures, it was clearly shown that NH_4^+ had a higher influence on protein content in cells when compared to NO_3^- [25]. It has also been proven that, in *Daucus carota*, the initiation of somatic embryogenesis cannot be started if the intracellular concentration in ammonium does not reach the minimal value of 5 mmol/g of fresh matter [26]. This minimum content cannot be reached without NH_4^+ in the medium.

Implication of Ca^{2+} in the initiation of somatic embryogenesis has been the subject of quite recent studies. To our knowledge, this study is the first one that provides evidence for the importance of Mg^{2+} in the initiation of somatic embryogenesis. The activation of protein synthesis must imply an increase of the enzymatic activity. With the multiple functions of Ca^{2+} and Mg^{2+} in enzymatic systems, it is conceivable that the initiation of embryogenesis is associated with specific requirement for these two cations. The modulation of intracellular concentration of Ca^{2+} is involved in the initiation of many processes of cellular differentiation [27]. In *Daucus carota*, it has been shown that the initiation of somatic embryogenesis is controlled by an increase in the concentration of Ca^{2+} [28]. Therefore, the specific uptake of Ca^{2+} during the initiation of somatic embryogenesis in *Cocos nucifera* could be attributed to an increase of the intracellular concentration.

Acknowledgements

We thank J. Chanut for his technical assistance and M. Noirot for his contribution to statistical analysis.

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