Specific Nutritional Requirements of Coconut Calli (*Cocos nucifera* L.) during Somatic Embryogenesis Induction

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Summary

Coconut calli were cultivated on two somatic embryogenesis induction media (SEIMs), differing in their 2,4-D content. Gain in dry matter weight, composition of soluble sugars within calli, but also pH and contents of glucose and macroelements in media were analysed at 0, 15, 28, and 60 days of culture. Relationships between contents of endogenous sugars, on the one hand, and between contents of media macroelements, on the other hand, were analysed. Comparison was made with calli maintained on a control multiplication medium.

Traits could be classified into 3 types of response with regard to condition of somatic embryogenesis induction (SEI condition).

The first correspond to traits that were modified by the SEI condition and varying over time. Two phases were determined. During the first phase (T0–T15), soluble sugar contents within calli decreased over time. The higher the 2,4-D content in SEIMs, the higher the sugar contents. Consumption of glucose and macroelements in media was negligible. However, strong relationships in the contents of chloride, nitrate, phosphate, and sulfate were modified in the SEI condition. During the second phase (T15–T60, growth became lower in the SEI condition. Requirements for glucose, nitrate and phosphate and acidification of media were higher. The relationship, determined by changes in nitrate and phosphate ($R > 0.98$), was modified by the SEI conditions, showing a preferential consumption for nitrate in this case. Endogenous sucrose content decreased to become lower in the SEI condition. The higher the 2,4-D content in SEIMs, the higher the requirements for media compounds, the higher the contents of sugars within calli, but the lower the growth.

The second type of response corresponded to traits modified by the SEI condition, but constant over time. It concerned relationships between contents of some cations in the media.

The third type of response corresponded to traits unchanges by the SEI condition and over time. It concerned the high relationship contents of endogenous glucose and fructose ($R = 0.88$), and between contents of chloride, ammonium, calcium, magnesium, and potassium.

Key words: Auxins, calli, carbohydrates, carbon source, Cocos nucifera L., cations, coconut, growth regulators, nitrogen requirement, phosphate requirement, somatic embryogenesis induction, sugar content, sulfate requirement, 2,4-D.

Abbreviations: ANOVA = analysis of variance; ANCOVA = analysis of covariance; BAP = 6-benzylaminopurine; DMW = dry matter weight; FMW = fresh matter weight; % WC = percentage of water content; SEIM = somatic embryogenesis induction medium; TFMW = total fresh matter weight.

Introduction

For different species, somatic embryogenesis induction has been correlated with several nutritional needs. Important carbohydrate requirements were known (Thorpe, 1983; Ammirato, 1987; Eapen and George, 1990), and a minimal nitrogen concentration was often necessary (Wheterell and Dougall, 1976; George and Sherrington, 1984; Ammirato, 1987; Vajrabhaya, 1988). However, these studies consisted essentially in testing different compound concentrations and in making a choice based on different criteria measured on the explant, such as the optimal growth or the number of embryogenic structures. Elaboration of the media is still more or less empirical.

By contrast, few studies have been devoted to the analysis of the consumption of nutritional components, which would be specific of one *in vitro* physiological step, such as somatic embryogenesis induction (Denchev et al., 1993; Dussert et al., 1995 b). With the determination of specific nutritional needs, such a study could lead to an optimisation of media composition and then to a better control of embryogenic events. In particular, this approach has been chosen for the recalcitrant species, coconut, for which somatic embryogenesis is often blocked or deviated (Blake, 1989; Verdeil et al., 1989; Buffard-Morel et al., 1992; Verdeil and Buffard-Morel, 1995). Several homogeneous coconut callus strains (Verdeil et al., 1994) that serve as models to study calli nutrition were established. By comparing calli in multiplication and somatic embryogenesis induction (SEI) conditions, specific higher requirements of calcium, magnesium and sucrose were described for different strains during embryogenesis induction (Dussert et al., 1995 b), which permitted a better adjustment of the media composition (Verdeil, 1993).

More recently, with other coconut callus strains, two different phases have been highlighted during somatic embryogenesis induction. The first ($\varphi 1$ phase) is the formation of embryogenic cells from meristematic cells during the first 15 days of culture on a somatic embryogenesis induction medium (SEIM) (Verdeil, 1993). It is defined as a latency phase as regards growth and adaptation to culturing. The second phase ($\varphi 2$ phase) occurred from the 15th to the 60th day of culture on the same medium, and corresponded to the formation of proembryos from embryogenic cells. This second phase was called the growth phase. These two phases have been associated with specific modifications in the amino acid composition of calli (Magnaval et al., 1995).

In the present study, our aim was not only to describe specific nutritional requirements of calli in the SEI conditions, but also to determine whether the two phases of embryogenesis induction could be distinguished on nutritional terms. This study was made by comparison with calli maintained in the multiplication condition. Furthermore, two somatic embryogenesis induction media (SEIMs), differing in their 2,4-D concentration, but exhibiting an equivalent efficacy towards embryogenesis induction (Verdeil, 1993), were tested. It allowed us to study whether nutritional requirements could be influenced by the 2,4-D level in SEIM. The growth of calli as well as their soluble sugar composition were analysed. Changes in pH, carbon source (glucose), and mineral element contents in the media were also studied.

Materials and Methods

Plant material

Plant material from the Marc Delorme Station (Abidjan, Côte-d'Ivoire) consisted of inflorescences sampled from 20- to 25-year-old hybrids (hybrid PB121 [West African Tall × Malayan Yellow Dwarf], copyright IRHO-CIRAD). Primary calli were obtained according to the callogenesis protocol described by Verdeil et al. (1994). Callus strain L82, described by Magnaval et al. (1995), was used here.

Culture media

Culture conditions of calli were described by Verdeil et al. (1989). Media contained: Murashige and Skoog's macronutrients modified by Rabechault and Martin ($mg \cdot L^{-1}$: KNO_3 2400, KH_2PO_4 1400, NH_4NO_3 2600, $CaCl_2$, $2H_2O$ 720, $MgSO_4$, $7H_2O$ 600) (Murashige and Skoog, 1962; Rabechault and Martin, 1976), Nitsch's micronutrients (Nitsch, 1969), Morel and Wetmore's vitamins (Morel and Wetmore, 1951), EDTA iron (EDTA: $26 mg \cdot L^{-1}$, $FeSO_4$, $7H_2O$: $24.9 mg \cdot L^{-1}$), glucose ($20 g \cdot L^{-1}$), ascorbic acid ($100 mg \cdot L^{-1}$), malic acid ($100 mg \cdot L^{-1}$), adenine sulfate ($30 mg \cdot L^{-1}$), BAP ($1 mg \cdot L^{-1}$), and agar agar ($7.5 g \cdot L^{-1}$). pH of media was adjusted to 5 prior to autoclaving (110 °C, 20 min).

Prior to treatment, the L82 clone was maintained on a multiplication medium (M100) containing $100 mg \cdot L^{-1}$ of 2,4-D and $3 g \cdot L^{-1}$ of activated charcoal.

Experimental design

Two factors (treatment and time) were considered.

Treatment involved a control medium (M100) and two somatic embryogenesis induction media (SEIMs). The latter is the control medium modified by twice the concentration of macronutrients, and a higher 2,4-D concentration: $130 mg \cdot L^{-1}$ and $140 mg \cdot L^{-1}$ in the presence of $3 g \cdot L^{-1}$ of activated charcoal (medium M130 and M140, respectively).

Four sampling dates, T0, T15, T28, and T60, were considered in a 60-day culture cycle.

For each «medium × date» combination, 11 tubes were considered: 1) 4 for growth measure, 2) 3 for the study of pH, mineral salts and glucose in media, and 3) 4 for endogenous sugar analyses. Each tube contained 200 mg of callus at the start of the experiment. The 132 tubes were placed in the culture room (temperature: 27 °C ± 1 °C, relative humidity: 55% ± 1%) according to a totally randomized design.

Growth measures

Each callus fresh matter weight was determined (FMW). Dry matter weight (DMW) was estimated from calli placed in a drying oven (48 h, 110 °C).

Gains, or losses, with regard to T0, the initial date, constitued the analyzed variables. On each «medium i × date j» combination, they were calculated by the substraction of DMW mean at T0 (200 mg ± 10 mg of calli) from each observed value.

Media glucose analysis

The glucose concentration in media was determined using the technique described by Fisher and Khotes (1951). The method was adapted as follows: 1) agar and activated charcoal were discarded from media by centrifuging samples for 15 min at 15,000 *g* and 2)

analysis was carried out on the filtered supernatant (Millipore: 0.45 µm) by a colorimetric method using dinitrosalicylate (or hydroxy-2 dinitro-3,5 benzoic acid). At 100 °C, glucose reduced dinitrosalicylate to a yellow-orange compound. Glucose was quantified by UV spectrophotometry at 510 nm by comparison with calibrated controls (Beulé, personal communication).

On each «medium i × date j» combination, gains or losses, with regard to initial date T0, were determined by the subtraction of the mean calculated on each medium type at T0 from each observed value. Results are expressed in $mg \cdot L^{-1}$ of medium.

Anion and cation analyses

Analysis of principal ions was performed by ion exchange chromatography (HPLC Dionex 4500i, Dionex Corporation, Sunnyvale, USA).

Anions (NO_3^-, SO_4^{2-}, Cl^- and $H_2PO_4^-$) were separated by an isocratic elution method (eluent: 3.9 mM of $NaHCO_3$ and 3.1 mM of Na_2CO_3) on a IONPAC ASSA column protected upstream by a guard column IONPAC AG5A.

Cations (NH_4^+, K^+, Na^+, Ca_2^+ and Mg_2^+) were separated on an IONPAC CS3 analytical column protected upstream by an IONPAC CG3 guard column. They were eluted by a «step-gradient» method: eluent was changed (from eluent 1 to eluent 2) 3 min after injection. Eluent 1 contained 12 mM HCl and 0.5 mM monochlorhydrate 2,3-diaminopropionic acid; eluent 2 contained 48 mM HCl and 8 mM monochlorhydrate 2,3-diaminopropionic acid.

Cations and anions were detected by conductimetry and quantified by comparison with control samples. Gains (or losses) were calculated as above. Results are expressed in $mg \cdot L^{-1}$ of medium.

pH estimation

The pH of media was measured in the supernatant.

Soluble sugar callus content analysis

Callus was weighed and divided approximately into 2 equivalent fractions. The first fraction was weighed, dried, and re-weighed for dry matter determination. The other fraction was weighed, frozen in liquid nitrogen, and stored at −30 °C until analysis. For analysis, the fraction was first crushed in liquid nitrogen. Soluble sugars were then extracted in 80 % alcohol in reflux (2 times 1 h), taken up in milli-Q water, evaporated on Rotavapor at 4 °C, and filtered (0.45 µm). They were then separated by ionic chromatography (HPLC DIONEX). The elution gradient was carried out for 40 min from 0 to 0.2 $mol \cdot L^{-1}$ NaOH. The flow rate was 1 $mL \cdot min^{-1}$. They were detected by pulsed amperometry. The nature and the concentration of sugars were determined with regard to calibrated controls. Results are expressed in mg per g of dry matter.

Statistical analysis

A two-way ANOVA (fixed model) was used to test effects of medium, time, and «medium × time» interaction. Planned comparisons using a contrast method permitted to test 1) the effect of the SEIM, and 2) the comparison between the two SEIMs.

Inter-media relationships between physiological traits were studied for each media using the covariance analysis (ANCOVA). When a relationship was significant, the squared-correlation coefficient R^2 was estimated to quantify the relative importance of the relationship. A parallelism test was also performed.

All statistical studies were performed using STATISTICA software.

Results

Initial characteristics of calli and media

At T0, calli were characterized by their dry matter weight (about 19 mg) and their sugar composition: glucose, fructose, and sucrose, with 2.9 %, 4.0 %, and 16.1 % of content, respectively.

At the beginning of the experiment, media were analysed for their pH, their glucose content, and their macroelements contents (Table 1). Glucose content and pH did not differ between media. The fact that SEIMs received a two-fold higher content of macroelements was taken into account to compare SEIM contents to the doubled values we observed on multiplication medium M100. In these conditions, cation contents did not change between media. By contrast, chloride and sulfate contents on SEIMs were lower than expected, whereas nitrate and phosphate contents were higher.

Changes of calli in the multiplication condition over 60 days

Gain of dry matter wras significant over time ($F_{3,12}$ = 42.3**). The highest gain was observed between T15 and T28, it then reached 50 mg in 60 days, about 2.5-fold the initial weight (Fig. 1 a). Calli changes over time concerned also their sucrose ($F_{3,12}$ = 7.66**), glucose ($F_{3,12}$ = 4.40*), and fructose ($F_{3,12}$ = 5.29*) contents. Sucrose content decreased strongly from T0 (160 $mg \cdot g^{-1}$) to T15 (60 $mg \cdot g^{-1}$), and increased from T15 to T28, in parallel with the high gain of dry matter. It then decreased to reach, at T60, a content 2-fold lower than at T0 (Fig. 1 b). Decrease of glucose and fructose contents differed from sucrose content fluctuations and were both very similar (Fig. 1 c and d). This agrees with the strong correlation (r = 0.97***) observed between

Table 1: Comparison of the pH level, and the contents of macroelements and glucose between media at the beginning of the experiment.

For contents of macroelements and glucose, results are expressed in mL per Liter of medium. In the case of macroelements, the F test was performed taking in account that Somatic Embryogenesis Induction Media (SEIMs) received a two-fold higher content (values observed on the multiplication medium were doubled for the analysis; their means are presented in the Table).

	Multiplication media[1]	SEIM M130[1]	SEIM M140[1]	Test F
Media pH	5.34	5.44	5.43	0.47 NS
Media Ca^{++}	85.1	179.3	197.3	1.17 NS
Media Mg^{++}	35.7	65.7	71.4	0.19 NS
Media K^+	536.7	1267.0	1310.7	0.30 NS
Media NH_4^+	209.3	487.0	537.3	0.23 NS
Media Cl^-	20.3	37.2	38.4	18.1**
Media NO_3^-	179.4	432.0	450.3	79.5***
Media $H_2PO_4^-$	54.7	113.8	119.9	43.1***
Media SO_4^{--}	16.4	27.4	28.2	60.2***
Media Glucose	21.2	23.3	23.8	2.46 NS

[1] Multiplication medium: M100 (100 $mg \cdot L^{-1}$ of 2,4-D; 3 $g \cdot L^{-1}$ of activated charcoal).

SEIMS: M130 (130 $mg \cdot L^{-1}$ of 2,4-D; 3 $g \cdot L^{-1}$ of activated charcoal); M140 (140 mg · L of 2,4-D; 3 $g \cdot L^{-1}$ of activated charcoal).

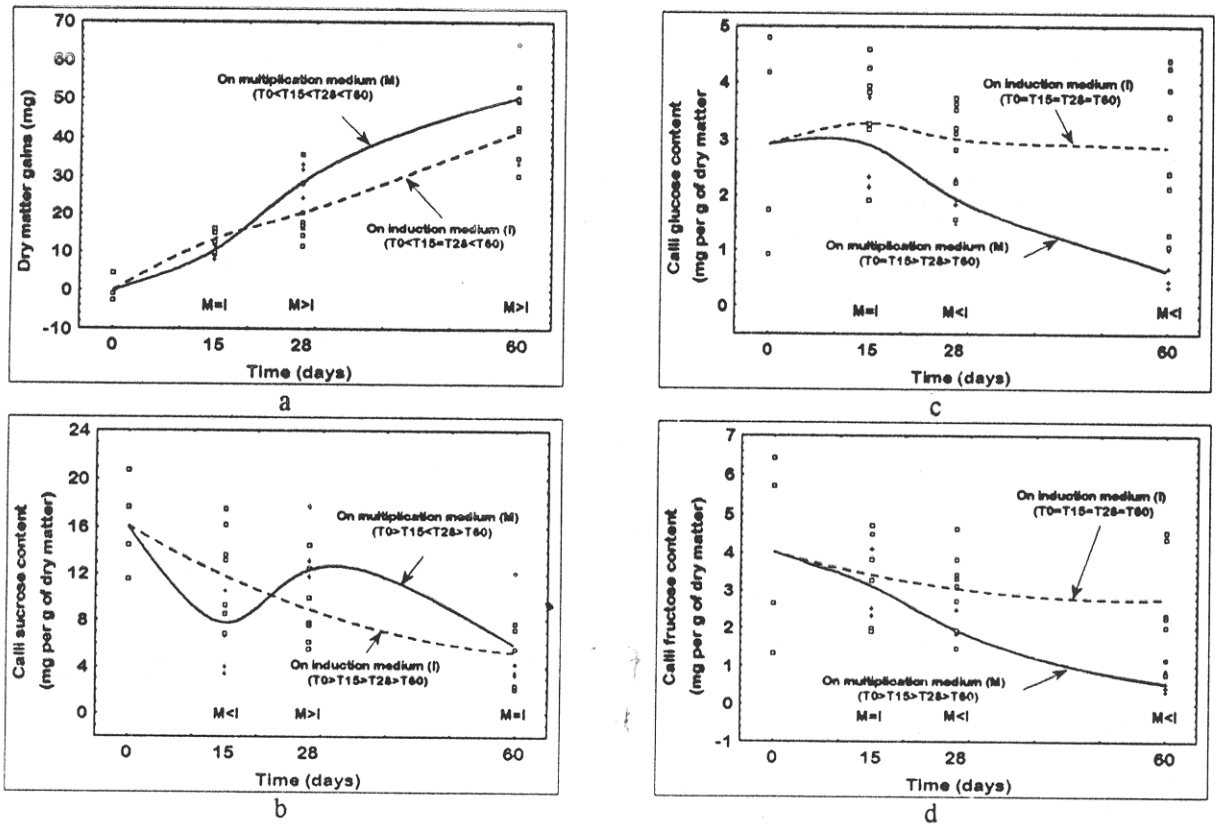

Fig. 1: Changes in dry matter (a), sucrose content (b), glucose content (c), and fructose content (d) of calli during subculture on multiplication medium (M) and somatic embryogenesis induction medium (I). Points represent samples for each interaction «medium × date». Results of statistical tests (planned comparison using a contrast method) were symbolized as M<I or T0 = T15<T28<T60, for example.

the two sugar contents. Significant, but weaker, correlations also existed between contents in sucrose and glucose ($r=0.44^{*}$) and in sucrose and fructose ($r=0.55^{*}$).

Changes in the multiplication medium over time

A regular decrease was observed after T15 for glucose ($F_{3,8} = 114.5^{***}$), nitrate ($F_{3,8} = 27.4^{***}$), phosphate ($F_{3,8} = 5.27^{*}$), and sulfate ($F_{3,8} = 57.8^{***}$) contents (Fig. 2 a to d). These changes were associated with a significant acidification of the medium (pH = 4.2 at T60) ($F_{3,8} = 13.2^{**}$) (Fig. 2 e). By contrast, some macroelement contents did not vary over time, e.g. as in the case of the cations (ammonium: $F_{3,8} = 2.09$; potassium: $F_{3,8} = 1.45$; magnesium: $F_{3,8} = 0.51$; calcium: $F_{3,8} = 0.66$) and the chloride anion ($F_{3,8}=0.34$).

More interesting were the co-variations observed between macroelement contents. Some correlations were shown by tyme-dependent macroelements, as in the case of the nitrate-phosphate ($r=0.98^{***}$), the nitrate-sulfate ($r=0.89^{***}$), and the phosphate-sulfate ($r = 0.86^{***}$) ratios. Changes in nitrate/phosphate ratio during the course of time are presented in Fig. 2 f. This ratio did not vary over time ($F_{3,8} = 0.72$). Similar results were noted for the sulfate/phosphate ratio ($F_{3,8} =.0.95$). The sulfate/nitrate ratio varied over time ($F_{3,8} = 4.36^{*}$), but only between T0 and T15.

Other correlations were seen with time-independent macroelements. The calcium-magnesium correlation was the strongest ($r = 0.89^{***}$). Weaker correlations existed between calcium and potassium ($r = 0.63^{*}$), between chloride and calcium ($r = -0.69^{*}$) and between chlorure and magnesium ($r = -0.72^{**}$). A correlation also existed between the time-dependent macroelement phosphate and the time-independent macroelement chloride ($r=0.62^{*}$).

Changes of calli in SEI conditions over 60 days

Observations on multiplication medium were compared with those on the two SEIMs. The gain of dry matter did not differ between M100 and SEIMs up to T15 and became lower later on SEIMs (T28: $F_{1,2^{*}} = 50.4$; T60: $F_{1,27} = 4.62$) (Fig. 1 a).

Effects of SEIMs also included a more regular decrease of sucrose content, in contrast with its fluctuations in multiplication medium (Fig. 1 b). Before T15, sucrose content was higher in SEI conditions than in multiplication conditions. After this date, content decreased and became lower in SEI conditions. The SEI conditions were also characterized by an absence of changes in glucose and fructose contents (Fig. 1 c and d). Nevertheless, relationships between sugar contents were not modified by SEI conditions.

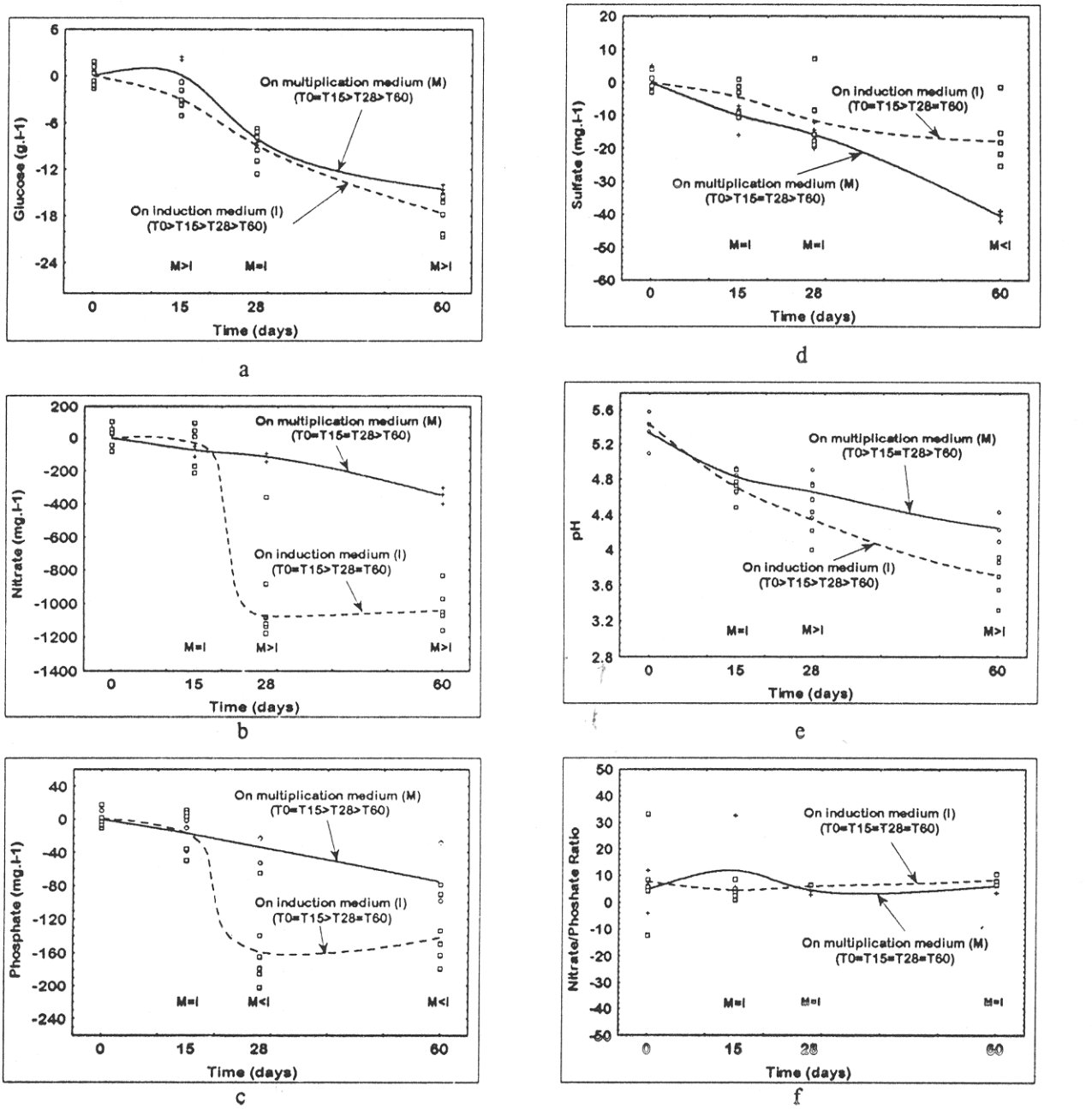

Fig. 2: Changes in nitrate content (a), phosphate content (b), sulfate content (c), «nitrate content/phosphate content» ratio (d), glucose content (e), and pH (f) in media during subculture on multiplication medium (M) and somatic embryogenesis induction medium (I). Points represent samples for each interaction «medium × date». Results of statistical tests (planned comparison using a contrast method) were symbolized as M<I or T0 = T15 < T28 < T60, for example.

Changes in somatic embryogenesis induction media over time

On SEIMs, glucose content decreased more regularly over time and consumption was slightly higher than on multiplication medium M100 (Fig. 2 a). This contrasted with the lower gain of dry matter by calli.

Two groups of macroelements can be distinguished from the comparison of SEIMs/M100.

The first group includes all cations and the chloride, that is time-independent ions. For this group, no differences were emphasized between SEIMs and M100.

The second group includes nitrate, phosphate, and sulfate, that is time-dependent ions. Two phases, φ1 and φ2, can be recognized (Fig. 2 b to d). Up to T15, (φ1), SEIMs and M100 were not different, whereas discrepancies appeared afterwards. In particular for nitrate and phosphate, changes

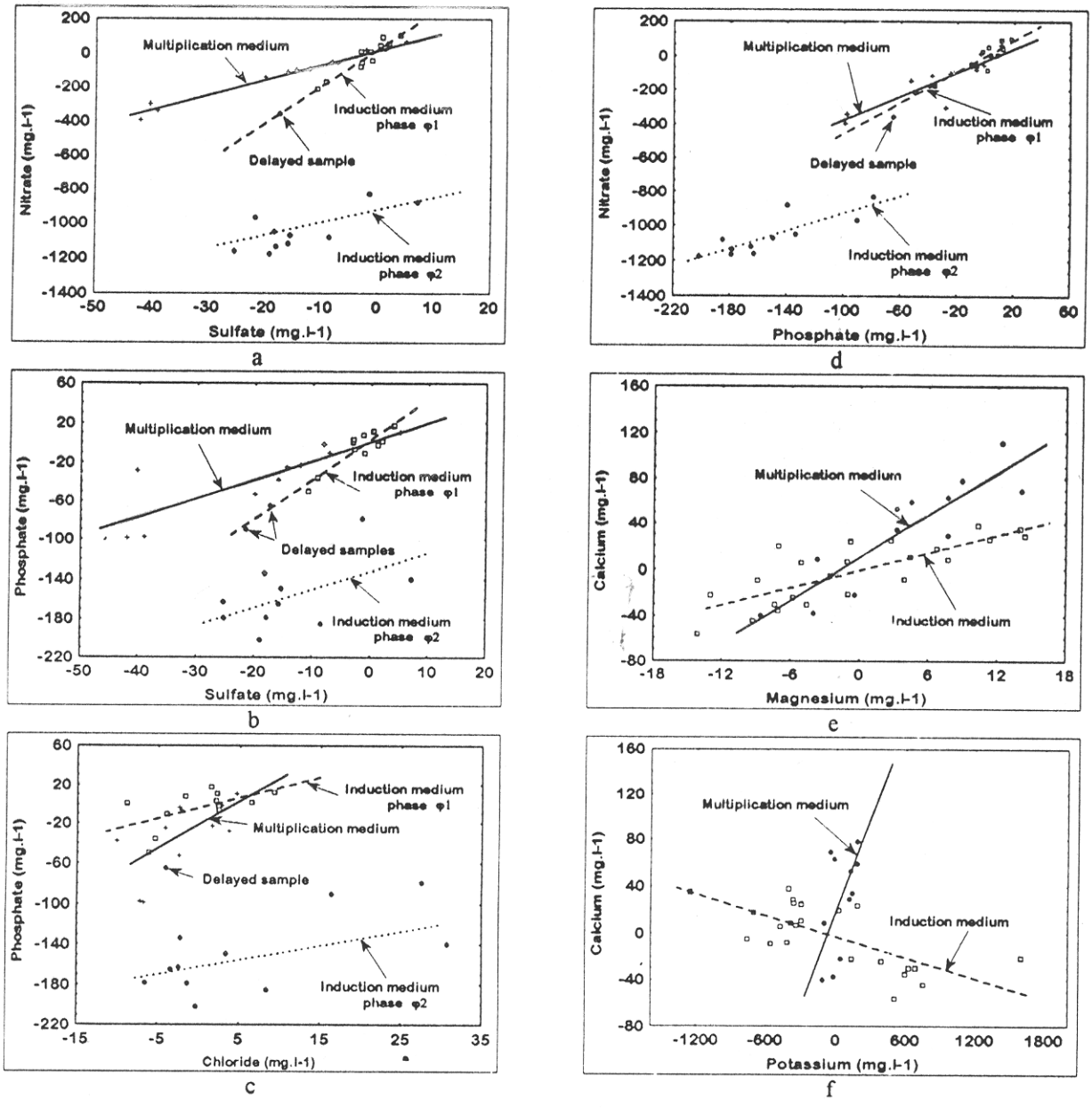

Fig. 3: Relationships between nitrate/sulfate (a), phosphate/sulfate (b), phosphate/chloride (c), nitrate/phosphate (d), calcium/magnesium (e), and calcium/potassium (f) contents in media during subculture on multiplication medium (M) and somatic embryogenesis induction medium (I). In the four first relationships (a to b), two phases can be distinguished on induction medium. The phase φ1 included T0 and T15, whereas, the phase φ2 included T28 and T60, except for one or two tubes at T28, which behaved as a T15 tube «delayed samples».

were very similar and the decrease was 5-fold greater in SEIMs.

In SEI conditions, another important effect was a marked change in relationships between some macroelements (Fig. 3). The φ1 and φ2 phases can be clearly distinguished for nitrate-sulfate, phosphate-sulfate, phosphate-chloride, and nitrate-phosphate relationships (Fig. 3 a to d). For the three former, differences in the multiplication conditions appeared as soon as the φ1 phase. Inter-sample heterogeneity was noted at T28. Indeed, two samples showed characteristics of the φ1 phase. They were called «delayed samples».

Other relationships were modified in SEI conditions without distinction of the two phases, e.g. in the case of calcium-magnesium and calcium-potassium relationships (Fig. 3 e and f). The strongest effect was seen for the latter, for which the positive relationship in multiplication conditions became negative in SEI conditions (Fig. 3 f).

For pH, presence of two phases was emphasized. SEIMs did not differ from multiplication medium the φ1 phase and became more acidic during the φ2 phase (Fig. 2 e).

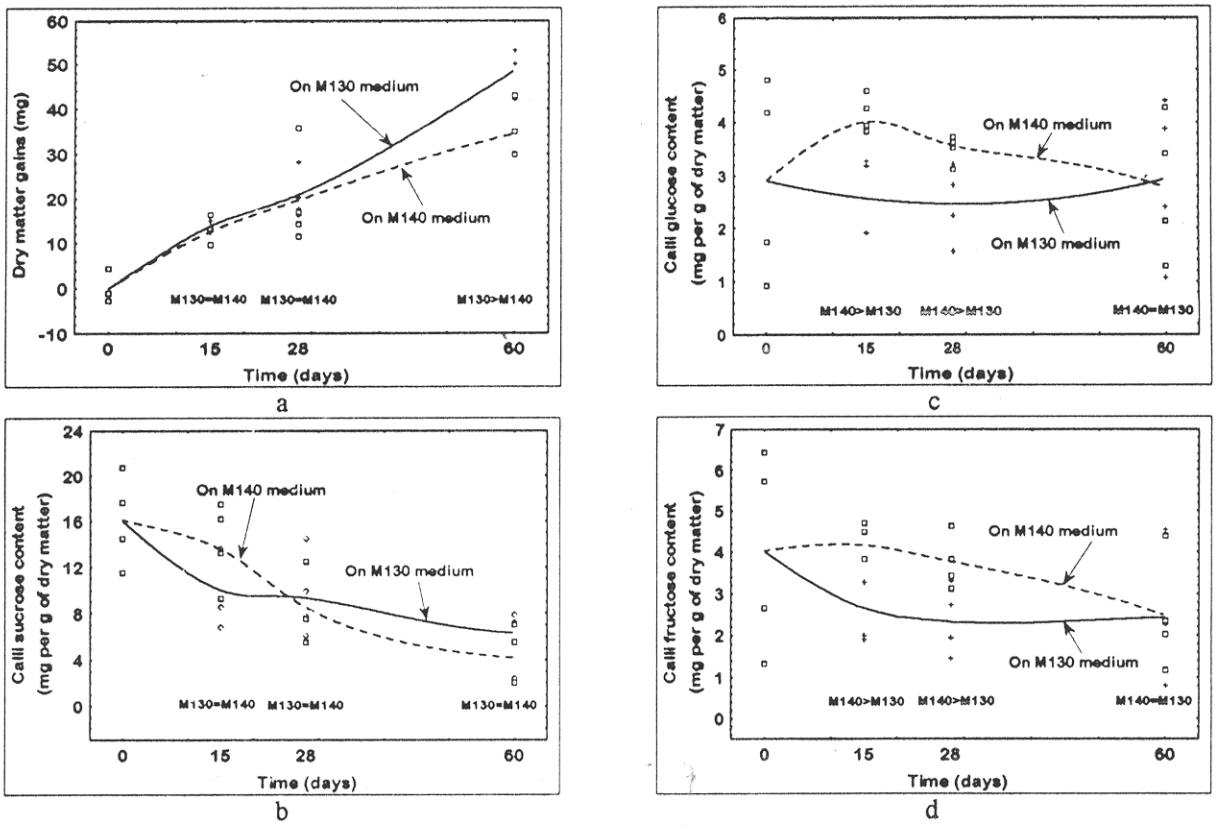

Fig. 4: Changes in dry matter (a), sucrose content (b), glucose content (c), and fructose content (d) of calli during subculture on the two somatic embryogenesis induction media. M130 (130 mg · L^{-1} of 2,4-D; 3 g · L^{-1} of activated charcoal) and M140 (140 mg · L^{-1} of 2,4-D; 3 g · L^{-1} of activated charcoal). Points represent samples for each interaction «medium × date». Results of statistical tests (planned comparison using a contrast method) were symbolized as M130 < M140, for example.

Effect of the 2,4-D content on calli in SEI conditions

Observations on the 2 SEIMs were compared.

The 2,4-D content changed the gain of dry matter only during the φ2 phase. Gain was lower on M140 (35 mg) than on M130 (49 mg) (Fig. 4 a). By contrast, glucose and fructose contents were higher on M140, although these effects disappeared at T60 (Fig. 4 c and d). Sucrose content was not modified by the 2,4-D content (Fig. 4 b).

Effect of the 2,4-D content on media changes in the SEI condition over time

The 2,4-D content influenced only glucose, phosphate, and nitrate contents (Fig. 5). The decrease of glucose content in media was higher at T28 on M140 and became lower at T60 (Fig. 5 a). Discrepancies between φ1 and φ2 phases for nitrate and phosphate contents were higher on M140 (Fig. 5 b and c). No effect was recorded on pH, cations, chloride, and sulfate contents, as well as on relationships between macroelements.

Discussion

The different traits studied can be classified into three types of responses to SEI conditions: 1) traits that were modified by SEI conditions and showing response variation; 2) traits that were modified by SEI conditions, but did not change over time; and 3) traits that were not modified by SEI conditions.

Traits modified by SEI conditions and varying over time

These traits confirmed the presence of two phases during embryogenesis (Magnaval et al., 1995).

The φ 1 phase of embryogenic cell formation

Variation during the φ1 phase was compared with the total variation over 60 days. In these conditions, nitrate, phosphate, sulfate, and glucose contents within media showed a weak or nill variation during the φ1 phase. As suggested previously (Magnaval et al., 1995), the φ1 phase could be considered as a latency phase of adaptation to culturing for consumption of media components.

However, two main types of changes appeared in SEI conditions as soon as the φ1 phase. The first concerned the higher contents of soluble sugars within calli. This could be related to higher requirements of carbohydrates during the formation of embryogenic cells, on coconut, as on other species (Thorpe, 1983; Ammirato, 1987; Eapens and George, 1990). These higher requirements could be used for: 1) the

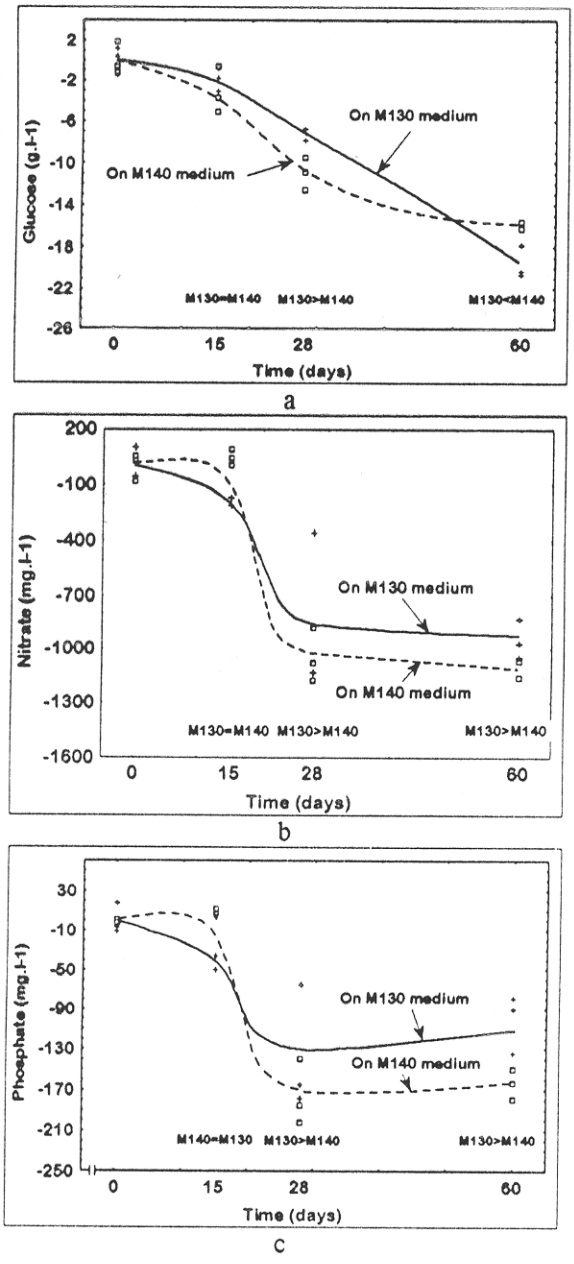

Fig. 5: Changes in glucose content (a), nitrate content (b), and phosphate content (c) in media during subculture on the two somatic embryogenesis induction media, M130 (130 mg · L^{-1} of 2,4-D; 3 g · L^{-1} of activated charcoal) and M140 (140 mg · L^{-1} of 2,4-D; 3 g · L^{-1} of activated charcoal). Points represent samples for each interaction «medium × date». Results of statistical tests (planned comparison using a contrast method) were symbolized as M130 < M140, for example.

thickening of the cell walls, observed when embryogenic cells begin their physical isolation; it could require the biosynthesis of further parietal polysaccharides (Michaux-Ferriere and Schwendiman, 1992; Verdeil et al., 1994), 2) the synthesis of the first starch reserves observed within embryogenic cells at the end of the φ1 phase (Magnaval et al., 1995).

Contents of endogenous sugars differed between the two SEIMs: the higher the 2,4-D content, the higher their contents. The precise role of exogenous auxins during somatic embryogenesis is still not well known enough to define here a direct relationship between these different compounds. However, as suggested by Taiz and Zeiger (1991), they could act like hormones during the development of the entire plant. A regulator effect of auxins on the synthesis of parietal polysaccharides, as cellulose (Ray and Abdul-Baki, 1968; Hall and Ordin, 1968) and proteins (Wightman and Setterfield, 1968; Dudits et al., 1991), was reported.

The second type of changes concerned relationships between nitrate and phosphate, sulfate and phosphate, and phosphate and chloride. The fact that contents of these anions were affected as soon as T0 (Table 1) suggests that changes should be independent of the embryogenic process.

In conclusion, the φ1 phase was not only a phase of adaptation to culturing, and could be considered as a phase of preparation for somatic embryogenesis. The induction treatment could cause a particular metabolic and bioenergetic cell-state that enables meristematic cells to switch on their defense mechanisms in a way that triggers the embryogenic pathway (Pedroso and Pais, 1995).

The φ2 phase of orientation towards proembryogenesis

Effects of SEI conditions were more important during the φ2 phase. In multiplication condition, the increase of endogenous contents in nutritive and energetic compounds could correspond to needs for calli growth and entry into a period of active cell divisions. By contrast, growth became lower in SEI conditions, whereas consumption of nutritive compounds (glucose, nitrate, and phosphate) was higher. At the beginning of the φ2 phase, embryogenic cells, which are physically isolated and accumulated reserves, did not divide (Verdeil, 1993). Cell divisions occur only after the 28th day of culture with the formation of the proembryos. The arrest of mitosis, during the formation of the embryogenic cell, could explain the lower DM weight in SEI conditions.

The lower increase of dry matter weight was accompanied by a higher consumption of glucose and phosphate within media, and a stronger decrease of endogenous sucrose content within calli in SEI conditions. This suggests a higher use of the nutritive compounds to produce energy necessary for the formation of proembryos (Yeung, 1995). The strong accumulation of reserves in embryonic cells (Magnaval et al., 1995) contrasting with the low increase of dry matter suggest that 1) the accumulation was specific to a low cell number becoming embryogenic, and 2) many other cells degenerated (Magnaval et al., 1995). In fact, the lower increase of dry matter could be explained by both a lesser mitosis rate and a higher cell degeneration. An enrichment of media with energetic compounds such as glucose and fructose should be expected from the process of cell degeneration. Absence of media enrichment suggests a higher squandering of energy.

Higher squandering of energy could be due to the restoration of damage caused by the higher content of 2,4-D in SEI conditions (George and Sherrington, 1984). Indeed, the higher the 2,4-D media content, the lower the DM weight. This result confirmed those obtained on other coconut callus

strains (Verdeil, 1993; Dussert et al., 1995 a). In many species, a high auxin level causes a stress on tissues growing in culture, leading to decreased growth (George and Sherrington, 1984). Furthermore, the higher the 2,4-D content in SEIM, the higher the squandering of energy (increase of the ratio ‹glucose consumption/dry matter gain›). This suggests a more important toxicity of 2,4-D at higher contents which could also explain cell degeneration.

Higher squandering of energy could also be due to a more expensive biosynthesis. In particular, the higher consumption of nitrate and the consequent modification of the relationship between nitrate and phosphate during the φ2 phase in SEI media could indicate a higher biosynthesis of amino acids and proteins. Indeed, these changes were concomitant with an increase of proline, valine, and leucine contents within calli (Magnaval et al., 1995), an activation of proteosynthesis (Verdeil, 1993) and an accumulation of protein within embryogenic cells and proembryos (Magnaval et al., 1995). An activated synthesis of these components was observed during embryogenesis induction in carrot (Fujimara and Komamine, 1982; Yeung 1995). This biosynthesis led to a new nutritional environment within embryogenic cells which will also be used for the formation and development of coconut proembryos and future embryos, and could be a prerequist condition for embryogenesis.

The two hypotheses of restoration of damage and more expensive biosynthesis could coexist simultaneously.

Traits modified by SEI conditions and constant over time

The second type of response observed within calli and media includes traits showing no change over time, but which differ between media conditions. This is the case in media with differing contents of time-independent ions, e.g. in relationships between calcium/magnesium and calcium/potassium. In particular, the last relationship, positive in the multiplication condition, became negative in the SEI conditions. The embryogenesis induction treatment could cause a disequilibrium of the ionic balance, as reported for calcium and potassium on *Camellia japonica* (Pedroso and Pais, 1995). However, considering the absence of variation in cation contents, this response could simply be related to undefined microvariations in media, independent of time, and similar to those observed between anions.

Traits unaffected by SEI conditions

The third of traits includes the relationship between glucose and fructose in calli as well as the contents of chloride, ammonium, calcium, magnesium, and potassium in media. The high ratio between glucose and fructose contents could reflect a constant equilibrium between biosynthesis and degradation of sucrose, depending on needs for carbohydrates (Wendler et al., 1990; Singh et al., 1991; Richter, 1993; Wang et al., 1993; Barnes et al., 1994). Chloride and potassium contents in media did not vary for other strains of coconut calli (Dussert et al., 1995 a). The absence of variation could be related to their role in the maintenance of turgor and ionic balance (George and Sherrington, 1984). The negligible changes in ammonium, calcium and magnesium contents contrasted with the higher requirements of these ions observed during embryogenesis for other strains of coconut calli (Dussert et al., 1995 b).

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