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Nutrient uptake and growth of in vitro coconut (*Cocos nucifera* L.) calluses

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Abstract

Growth of two strains, L1 and L7, of coconut calluses in vitro and changes in concentration of major nutrients and sugars in the media were measured after 0, 14, 28, 42 and 56 days of culture. A histological check was performed on days 0, 28 and 56. Although the calluses were multiplied on two different concentrations of 2,4-D, growth (dry weight) was identical for both strains and was linked to the same nutrient uptake. Organization of the meristematic zone of the calluses in the two strains was very different, but had no influence on their nutrition. NH_4^+, Ca^{2+} and Mg^{2+} uptake occurred throughout the culture period. Sucrose and SO_4^{2-} were absorbed only during the lag phase of growth and at the beginning of the exponential growth phase. $H_2PO_4^-$ and NO_3^- uptake occurred only during the second half of the exponential phase. Ionic balance was maintained by K^+ and Cl^-.

Keywords: Arecaceae; Growth; Mineral nutrients; Sugar; Callus

1. Introduction

In all in vitro techniques, the choice of organic and mineral composition of the culture medium determines the success of the method. Indeed, for any growth or cell differentiation process, the specific requirements depend on the species studied, the nature of the tissue, and its physiological state. Therefore, it is particularly important to know these specific requirements for each step of the in vitro vegetative propagation technique under consideration.

With coconut palm, somatic embryogenesis is the most commonly studied technique, but the regeneration of whole plants has not been sufficiently perfected so far to ensure the production of plantlets on a large scale [1]. Besides, the coconut is considered by many authors as a recalcitrant plant [1,2]. The heterogeneity of primary calluses is partly responsible for the difficulty of perfecting a regeneration method.

Numerous strains of calluses, which constitute a

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

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very homogeneous material, have been established in our laboratory [3,4]. Optimal growth of two of these strains, L1 and L7, is sustained by two different concentrations of 2,4-D. Moreover, a histological check shows that the organization of the meristematic zone of the calluses differs considerably between the strains.

Two approaches can be distinguished in the study of the nutritional requirements of a tissue at a definite state. The first classical approach is to vary the concentration of the nutrients in the medium and make a choice on the basis of a few criteria measured on the studied explant. Numerous media have been defined using that approach: George and Sherrington [5] have listed more than 260, as well as 150 mineral solutions. The second approach is to measuring nutrient uptake from the medium. This approach has been used in very few studies [6–17] and has been used in this study. We measured the changes in concentration of all major elements and sugars in the media.

2. Materials and methods

2.1. Plant material

Experiments were conducted using two strains of coconut calluses, L1 and L7, obtained from immature floral meristems taken from an adult tree (20 years old), Malayan Yellow Dwarf × West African Tall hybrid (hybrid PB121, IRHO/CIRAD, Ivory Coast). Primary calluses were obtained according to the callogenesis protocol described by Verdeil et al. [4]. The two strains, L1 and L7, were established by multiplying the primary calluses, on M3 and M2 media, respectively. Calluses were kept in the dark at 27 ± 1°C and subcultured every 2 months.

2.2. Culture media

The medium contained Murashige and Skoog [18] macronutrients modified as follows: 1200 $mg \cdot l^{-1}$ KNO_3; 700 $mg \cdot l^{-1}$ NH_4NO_3; 360 $mg \cdot l^{-1}$ $CaCl_2$, $2H_2O$; 300 $mg \cdot l^{-1}$ $MgSO_4$, $7H_2O$; Murashige and Skoog [18] minor elements, Morel and Wetmore [19] vitamins, 40 $mg \cdot l^{-1}$ adenine sulphate, 30 $g \cdot l^{-1}$ sucrose, 2 $g \cdot l^{-1}$ activated charcoal and 7.5 $g \cdot l^{-1}$ agar (Sigma). Media M2 and M3 contained 2.7×10^{-4} M and 3.6×10^{-4} M 2,4-D, respectively. pH was adjusted to 4.5 with 1 N H_2SO_4 prior to adding agar and activated charcoal. Aliquots (20 ml) were dispensed in 2.4 × 18 cm test tubes sealed with Parafilm Ribbon. Media were autoclaved at 110°C and 103 KPa for 20 min.

2.3. Inoculation and culture conditions

Calluses used for the inoculation and test tubes were randomized. Up to 200 ± 20 mg (fresh weight) of callus were inoculated. In a first experiment, calluses of both L1 and L7 strains were cultured on media M2 and M3 to determine the optimal growth medium. In all other experiments, calluses of the L1 strain were cultured on medium M3 and those of the L7 strain on medium M2. Cultures were maintained for 56 days in the dark at 27 ± 1°C and 55 ± 2% relative humidity.

2.4. Measurements

Measurements were taken on days 0, 14, 28, 42 and 56. Each set of conditions (2,4-D concentration × day or strain × day) was represented by four samples. At sampling time, fresh weight and dry weight were measured for each callus. The dry weight was determined after drying for 48 h at 100°C.

Eight control test tubes with no explant were weighed at each sampling time in order to estimate the average dehydration of the culture media. This estimation was used to correct the measured sugar and macronutrient concentrations.

After removal of callus, the pH of the medium was determined by inserting the electrode of the pH meter directly into the medium. Media were then stored at −20°C. For ion and sugar concentration measurements, media were thawed and centrifuged for 20 min at 15 000 × *g*. The supernatant was recovered and stored at −20°C. Measurements were made on the thawed supernatant.

Major element concentrations were measured by HPLC (Dionex 4500i, Dionex Corporation, Sunnyvale, USA) using a conductivity detector. Anion concentrations were measured using an isocratic method (eluant: 3.9 mM $NaHCO_3$, 3.1 mM Na_2CO_3; Guard Column IONPAC AG5A; Analytic Column IONPAC AS5A). For cations, a step-gradient was applied by changing the eluant

three minutes after injection (eluant 1, 12 mM HCl and 0.5 mM 2,3-diaminoproprionic acid monohydrochloride; eluant 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 by the Bergmeyer and Bernt [20] enzymatic method (Beckman DU 70 spectrophotometer, Fullerton, USA). Three measurements were made for each sugar and each sample.

An histological check was performed according to the method described by Buffard-Morel et al. [3] on days 0, 28 and 56.

2.5. Statistical analysis

In the figures, each point corresponds to the average value and smoothing of the sequences was obtained by Moving Polynomial Regression [21].

A two-way analysis-of-variance was applied to test the effects of time, 2,4D concentration, and the time-2,4-D concentration interaction on fresh weight of calluses. A two-way analysis-of-variance was applied to test the effects of time, strain, and the time-strain interaction on dry weight of calluses, pH of the media, and changes in the concentration of major nutrients and sugars in the media. Time, 2,4-D concentration, and strain were fixed-effect factors. When an effect was significant, the Newman and Keuls' test was used for multiple comparison of categorial means [22,23]. For the main results, we give the observed (Q_{obs}) and expected (Q_{exp}) values of Q (Q_{exp} comes from Newman and Keuls' table for $P = 0.05$).

On each graph, bars indicate the standard deviation estimated from the square-root of the residual mean square of two-way ANOVA.

The whole experiment was performed in duplicate and the results obtained were similar.

3. Results

3.1. Statistics

For all the traits studied, except fresh weight, two-way ANOVA showed that there was no strain effect and no time-strain interaction (Table 1). By contrast, there was always a time effect.

Table 1
Results of two-way analysis-of-variance

Trait	Strain effect $F_{30\text{-}1}$	Time effect $F_{30\text{-}4}$	Time-strain interaction $F_{30\text{-}4}$
Dry weight	0.00[a]	16.3[d]	1.02[a]
Sucrose	2.98[a]	25.84[d]	1.93[a]
Glucose	1.09[a]	3.17[b]	0.64[a]
Fructose	1.94[a]	3.64[b]	0.83[a]
pH	0.09[a]	51.6[d]	1.98[a]
$H_2PO_4^-$	0.17[a]	12.03[d]	1.92[a]
NO_3^-	0.25[a]	12.31[d]	0.83[a]
SO_4^{2-}	0.09[a]	23.15[d]	1.10[a]
NH_4^+	2.73[a]	191.7[d]	1.91[a]
Mg^{2+}	0.00[a]	41.52[d]	0.32[a]
Ca^{2+}	0.88[a]	42.74[d]	0.84[a]
K^+	1.74[a]	6.73[c]	1.93[a]
Cl^-	0.11[a]	4.97[c]	0.33[a]

For each effect tested and trait studied, F-value is given as follows: [a]Not significant; [b]Significant at $P < 0.05$; [c]Significant at $P < 0.01$; [d]Significant at $P < 0.001$.

3.2. Histological check

Callus growth on multiplication medium was ensured, in both strains, by peripheral meristematic zones. In strain L1, this meristematic zone was organized in a cambium-like structure, whereas in strain L7 it was disorganized (Fig. 1). In the internal area of the L7 calluses, cells were differentiated into parenchymatous tissue. A few

Table 2
Results of two-way analysis-of-variance

Effect	Strain	
	L1	L7
2,4-D concentration effect $F_{30\text{-}1}$	5.13[b]	7.22[b]
Time effect $F_{30\text{-}4}$	17.30[c]	19.82[c]
Time-strain interaction $F_{30\text{-}4}$	1.24[a]	0.93[a]

For each effect tested and trait studied, F-value is given as follows: [a]Not significant; [b]Significant at $P < 0.01$; [c]Significant at $P < 0.001$.

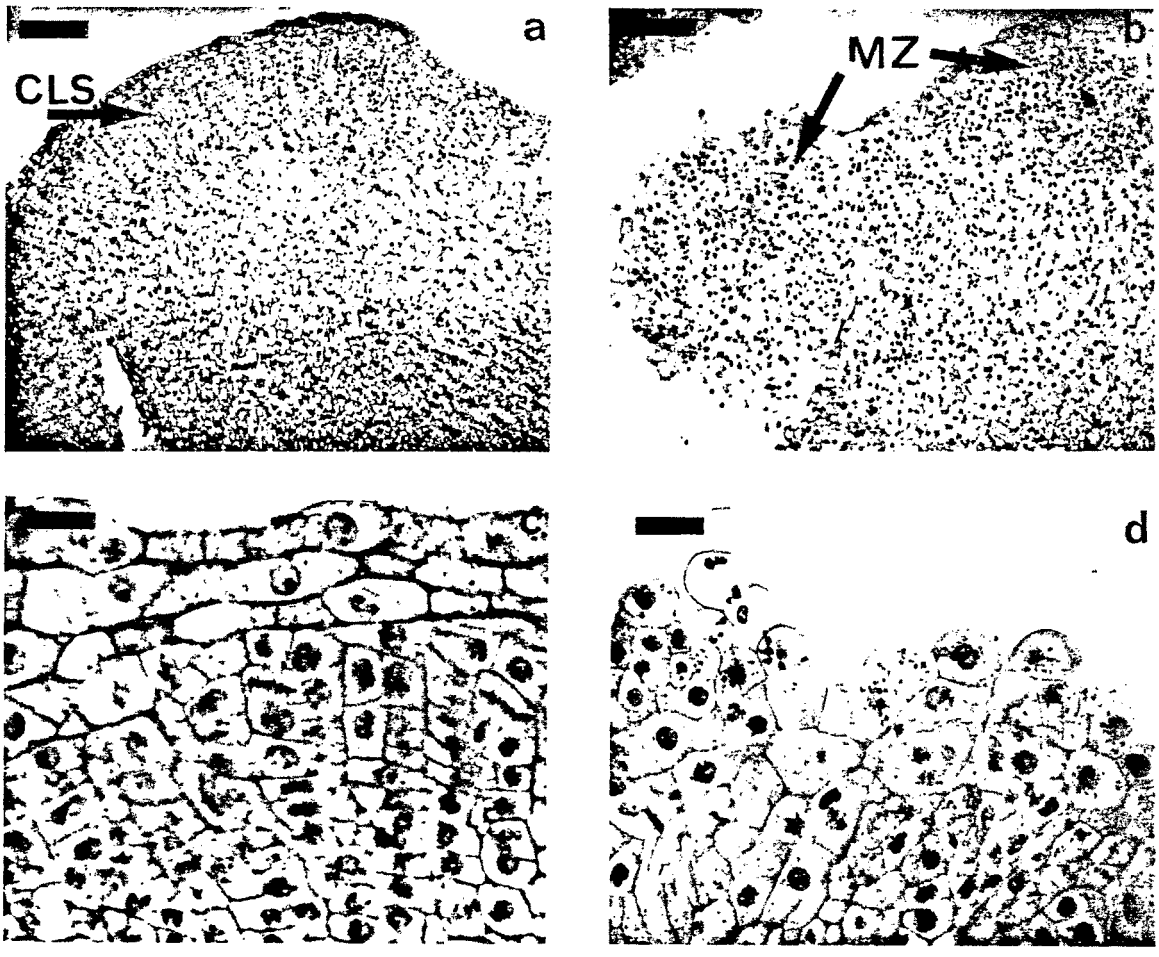

Fig. 1. (a) L1 callus strain: callus growth ensured by a peripheral meristematic zone organized in a cambium-like structure (CLS) (bar = 190 μm) (b) L7 callus strain: callus growth ensured by a non-organized peripheral meristematic zone (MZ) (bar = 170 μm). (c,d) Details of the peripheral meristematic zone in L1 callus (c, bar = 40 μm) and in L7 callus (d, bar = 55 μm).

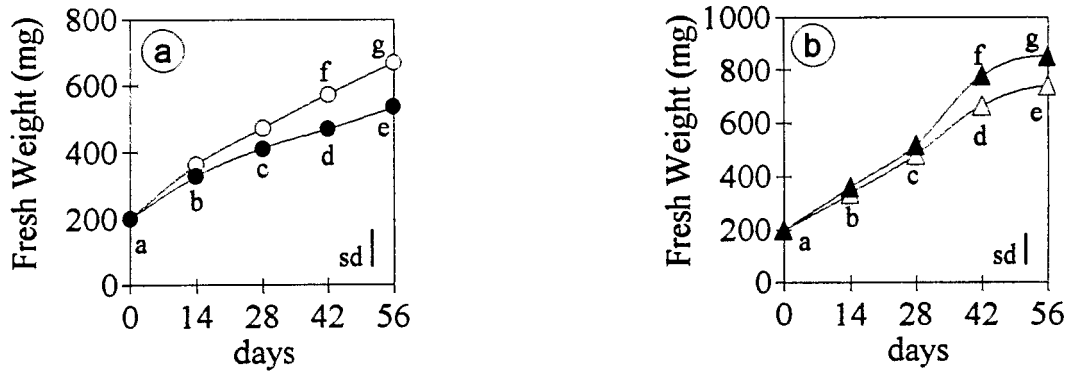

Fig. 2. Changes in the fresh weight of calli of strains L1 (a) and L7 (b) on M2 (●, ▲) and M3(○, △) medium. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test (sd, standard deviation).

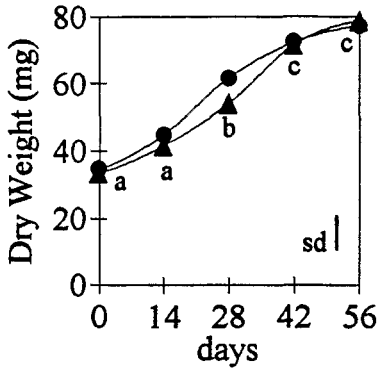

Fig. 3. Changes in the dry weight of calli of strains L1 (●) on M3 medium and L7 (▲) on M2 medium. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test (sd, standard deviation).

tracheids were present in the internal zone of L1 calluses.

3.3. Growth

For both strains, there was a significant effect of 2,4-D concentration and time (Table 2). Thus, optimal growth was achieved with 3.6×10^{-4} M 2,4-D (M3) for strain L1 and 2.7×10^{-4} M 2,4-D (M2) for strain L7 (Fig. 2a,b).

There was no significant difference in dry weight between strain L1 on medium M3 and strain L7 on medium M2, either between days 0 and 14 ($Q_{obs} = 1.87$; $Q_{exp} = 2.88$), or between days 42 and 56 ($Q_{obs} = 1.99$; $Q_{exp} = 2.88$). Thus, one can infer that growth (dry weight increase) started about day 14 and stopped about day 42 (Fig. 3).

3.4. Sugars

Less than 2% of sucrose was hydrolyzed during autoclaving (Fig. 4a). Two-thirds of the sucrose absorbed was taken up during the first 14 days. There was no significant difference between day 28 and day 56 in sucrose concentration ($Q_{obs} = 0.19$; $Q_{exp} = 3.47$). After 56 days in culture, 7 $g \cdot l^{-1}$ sucrose were left in the media, representing 25% of the initial concentration. The glucose concentration in the media increased significantly during the first 28 days up to 2 $g \cdot l^{-1}$ ($Q_{obs} = 5.11$; $Q_{exp} = 3.47$), and then stopped increasing ($Q_{obs} = 0.84$; $Q_{exp} = 3.47$) (Fig. 4b). The fructose concentration increased from 0.5 to 2.5 $g \cdot l^{-1}$ in the first month of culture ($Q_{obs} = 6.48$; $Q_{exp} = 3.47$), and then decreased to 0.5 $g \cdot l^{-1}$ after 56 days in culture ($Q_{obs} = 4.73$; $Q_{exp} = 3.47$) (Fig. 4c).

3.5. Changes in pH

The initial pH was set to 4.5 prior to adding agar and activated charcoal. It was 5.8 after autoclaving (Fig. 5a). The pH decreased to 5.2 on day 14 ($Q_{obs} = 23.66$; $Q_{exp} = 2.88$), to 5 on day 28

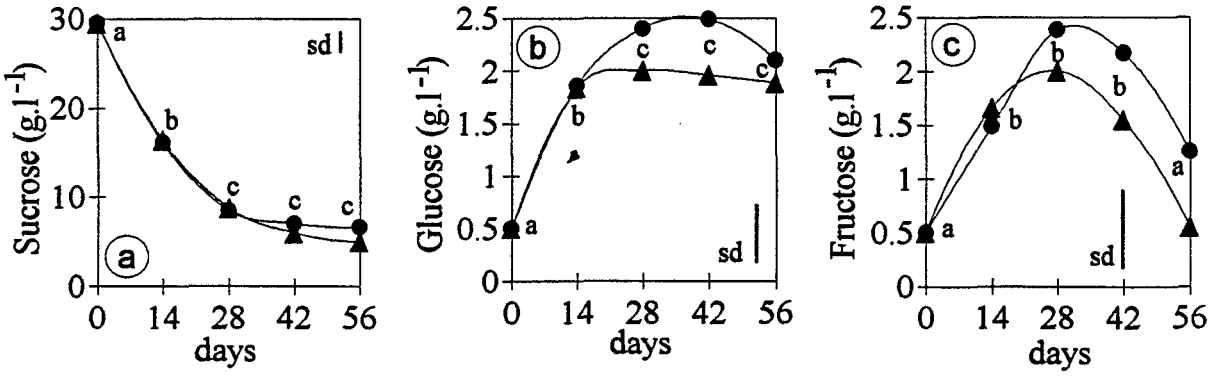

Fig. 4. Changes in the concentrations of sucrose (a), glucose (b) and fructose (c) in medium M3 for strain L1 (●) and in medium M2 for strain L7 (▲). Points followed by the same letter are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test (sd, standard deviation).

(Q_{obs} = 5.58; Q_{exp} = 2.88), to 4.8 on day 42 (Q_{obs} = 6.81; Q_{exp} = 3.47), and to 4.7 after 56 days in culture (Q_{obs} = 6.06; Q_{exp} = 2.88).

3.6. Major nutrients

NH_4^+ and Ca^{2+} uptake occurred throughout the culture period (Fig. 5b,c). There was no significant uptake of NO_3^-, $H_2PO_4^-$ and Mg^{2+} after day 42 (Q_{obs} = 1.46, 1.77 and 1.37, respectively; Q_{exp} = 2.88) (Fig. 5d,e,f). There was no significant uptake of NO_3^- before day 28 (Q_{obs} = 2.28; Q_{exp} = 3.47). $H_2PO_4^-$ uptake started from in-

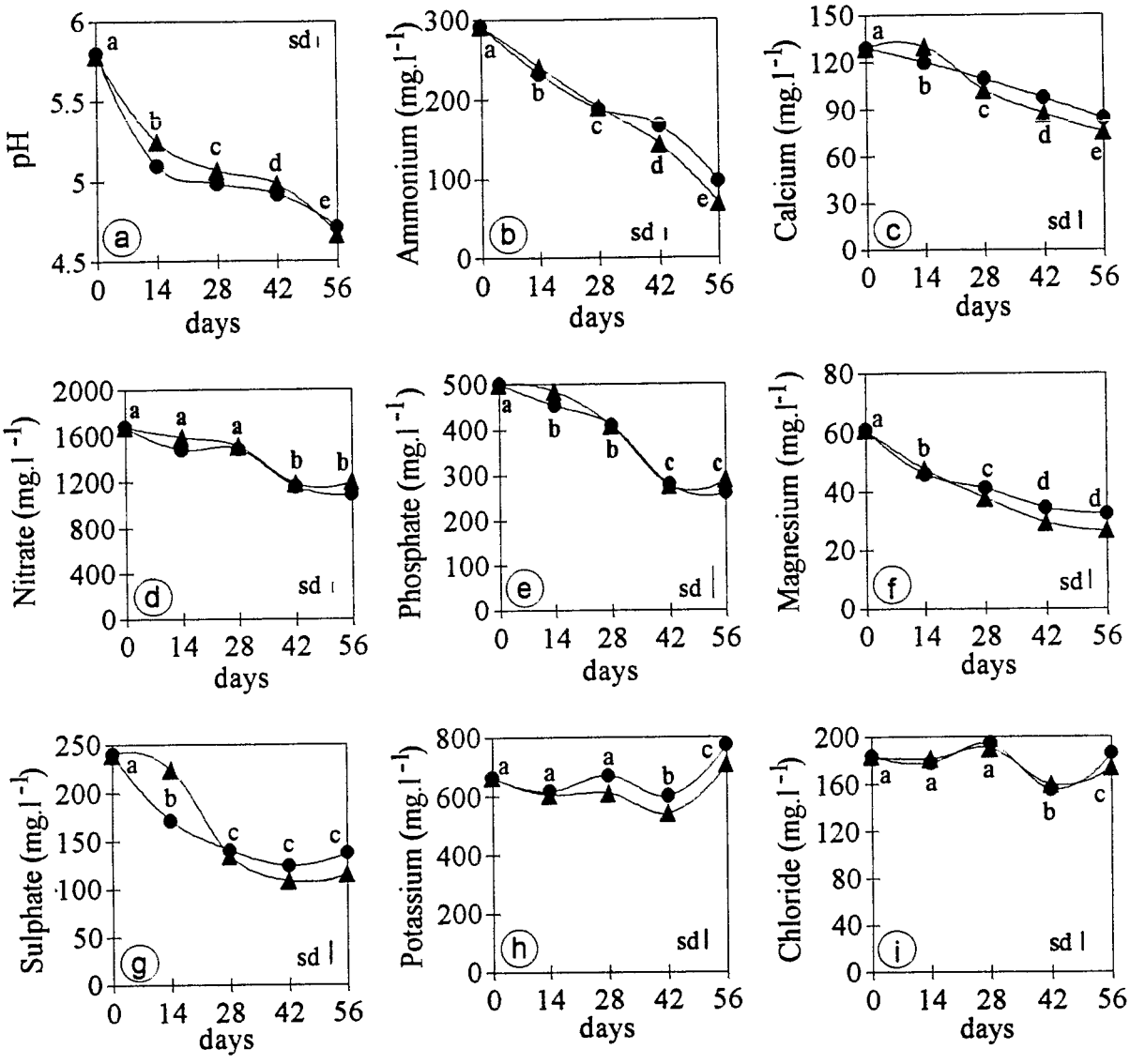

Fig. 5. Changes in the pH (a) and in the concentrations of major nutrients (b, ammonium; c, calcium; d, nitrate; e, phosphate; f, magnesium; g, sulfate; h, potassium; i, chloride) in medium M3 for strain L1 (●) and in medium M2 for strain L7 (▲). Points followed by the same letter are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test (sd, standard deviation).

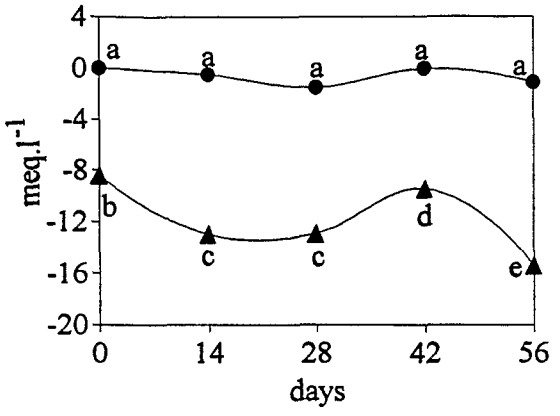

Fig. 6. Changes in total ionic charges in the medium with (●) or without (▲) K^+ and Cl^-. Points followed by the same letter are not significantly different at the 0.05 probability level as determined by the Newman and Keuls' test (sd, standard deviation).

oculation but occurred mainly between day 28 and day 42. SO_4^{2-} uptake started from inoculation and stopped at day 28 (Q_{obs} = 2.38; Q_{exp} = 3.47) (Fig. 5g). K^+ and Cl^- were significantly absorbed between days 28 and 42 (Q_{obs} = 5.98 and 4.48, respectively; Q_{exp} = 2.88), but were extruded afterwards up to the baseline for Cl^- (Q_{obs} = 0.0013; Q_{exp} = 4.07) and above baseline for K^+ (Q_{obs} = 4.48; Q_{exp} = 4.07) (Fig. 5h,i).

The total ionic charges without Cl^- and K^+ fluctuated significantly throughout the 2 months of culture (F = 5.57, P = 0.0060), whereas with K^+ and Cl^- the total ionic balance was stable (F = 0.93, P = 0.4715) (Fig. 6).

4. Discussion

Strains L1 and L7 can be distinguished in two main ways. Their optimal growth was controlled by two different concentrations of 2,4-D. Their calluses also differed in the level of organization of the meristematic zone. In spite of these differences, mineral and sugar requirements seemed quantitatively and kinetically equivalent. It is particularly interesting to note that the maximal meristematic activity led to identical production of dry matter, although it was controlled by a different 2,4-D concentration and involved equivalent requirements independent of meristematic organization.

During the first 14 days, there was a rapid decline in sucrose in the medium, but no significant increase in dry weight. Thus, one can assume that during the lag phase the greater part of the absorbed sucrose is used for energy production. High respiration rate during the lag phase has been reported by Maretzki et al. [24]. This phase has often been described as a preparatory period of cell division [5]. Hence, the increase in intracellular concentration of energetic compounds may play a role in the preparation of the mitotic phase.

The increase in glucose and fructose levels in the medium during the first month of culture indicates extracellular hydrolysis of sucrose. In some species, the sucrose is entirely hydrolyzed before being absorbed [17,25,26]. However, it has never been demonstrated, except for sugar-cane parenchyma [25], that hydrolysis is strictly required for absorption.

Two results are notable concerning the nitrogen nutrition of coconut calluses: preferential use of NH_4^+ over NO_3^- at the beginning and end of the culture, and changes in pH of the media.

During the first month of culture, there was no significant uptake of NO_3^-, whereas NH_4^+ was absorbed from inoculation onwards. This preferential uptake of NH_4^+ at the beginning of culture has been observed for many species when the initial pH of the medium was between 5.5 and 6.5 [8,13,17,27]. In *Ipomea* sp. cell culture, NH_4^+ was absorbed in preference to NO_3^- at the beginning of culture if the pH was 6.3 [28]. In contrast, NO_3^- uptake occurred prior to NH_4^+ uptake if the initial pH was 4.8. It was reported that NH_4^+ uptake acidifies the medium and thus indirectly promotes NO_3^- uptake [5]. The decrease in pH during the first 14 days would thus derive from NH_4^+ uptake. In rice suspensions culture, the decrease in pH during the first hours of incubation was caused by the uptake of NH_4^+ and, in the following hours, the pH increased during absorption of NO_3^- [17]. In our study, preferential uptake of NH_4^+ also occurred during the last 14 days. This result cannot be attributed to extracellular pH. Hence, too high an intracellular concen-

tration of ammonium could explain this final preferential uptake by inhibiting both nitrate and nitrite reductase activity. This inhibition was shown in *Ipomea* cell culture [27]. It was also clearly demonstrated that too high an uptake of NH_4^+ had a toxic effect on rice cells since they can not regulate the absorption of NH_4^+ [17].

$H_2PO_4^-$ was not absorbed before day 28. Usually, $H_2PO_4^-$ is the nutrient most rapidly absorbed after inoculation [8,10,12,13,17,27]. It is unexpected that $H_2PO_4^-$ was not used during the first 14 days, an intensive energy-producing period. The initial pH of the medium could be the limiting factor [30]. In rose cell suspensions, whatever the initial phosphate concentration in the medium, the depletion of phosphate corresponded in time to the rapid fall in pH of the medium [6]. Sentenac and Grignon [31] showed that $H_2PO_4^-$ uptake in corn roots was minimal at pH 6 and nearly maximal at pH 5. With *Petunia* cells cultured in vitro, optimum phosphate uptake occurred at pH 4, and declined rapidly when the pH was raised [32].

According to Mengel and Kirby [33], the different roles of K^+ can be summed up by the maintenance of turgor and ionic balance. Our experiments show the function of K^+ in ionic balance. Cl^- is generally considered an inert anion [34]. During K^+ fluxes, its function would be to maintain the ionic balance by fluxes oriented in the same direction as K^+ [35]. Thus, changes in the concentrations of K^+ and Cl^- follow similar patterns.

This study pointed out some nutritional mechanisms in connection with in vitro callus growth: sucrose hydrolysis, influence of the pH of the medium on NO_3^- and $H_2PO_4^-$ uptake, influence of nitrogenous compound uptake on extracellular pH, and importance of K^+ and Cl^- in the maintenance of ionic balance.

Comparison of the nutritional requirements of the coconut calluses during growth and initiation of somatic embryogenesis could reveal some interesting relationships.

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