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Free Amino Acid Composition of Coconut (*Cocos nucifera* L.) Calli under Somatic Embryogenesis Induction Conditions

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Summary

The amino acid composition of coconut calli under somatic embryogenesis induction conditions was compared with that of calli under multiplication conditions. Six amino acids were detected: proline, valine, leucine, serine, alanine and threonine. Calli were classified into five groups according to their amino acid composition by a clustering method taking into account the morphogenetic stage of each callus. A discriminant analysis and an analysis of variance revealed that the groups had been classified correctly. The first group comprised all calli sampled at the beginning of culture (T0). Thereafter, two major phases were detected. The first phase concerned analysis times T8 and T15 and corresponded to a latency phase as regards growth and adaptation to culture conditions. Calli on the multiplication medium were significantly different from calli on the induction medium, characterized by a drop in proline, valine and serine contents. This drop was an initial reaction to the increase in 2,4-D and mineral element concentrations in the media to initiate unicellular type embryogenesis. The second phase, regrouping analysis times T28, T42 and T60, corresponded to callus growth. Heterogeneity was detected in calli on the induction medium and was confirmed by a histological study. Some calli adapted to the changes in the medium by remaining at the meristematic stage; their amino acid composition became similar to that of calli on the multiplication medium. The others tended well towards embryogenesis and were characterized by increases in the proline, valine and leucine contents and an unchanged alanine content; these increases were concomitant with the formation of storage proteins observed histologically at the time of embryogenesis initiation.

Key words: Calli, Cocos nucifera L., clustering method, free amino acids, histological study, morphogenetic stage, somatic embryogenesis initiation.

Abbreviations: CM = clustering method; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6-benzylaminopurine; Ala = alanine; Leu = leucine; Pro = proline; PTC = phenylisothiocyanate; SEIM = somatic embryogenesis initiation medium; Ser = serine; Thr = threonine; Val = valine.

Introduction

The search for endogenous metabolic signals in order to estimate the physiological status of calli at the first steps of somatic embryogenesis is a worthwhile approach for species such as coconut, for which embryogenic deviation is often observed (Blake, 1989; Verdeil et al., 1989; Buffard-Morel et al., 1992). Some endogenous amino acids have been shown to be involved in differentiation and morphogenesis. Thorpe (1983) noted an accumulation of proline, serine and threonine during bud formation on tobacco calli. Likewise, glutamate and serine disappear from castor-oil plant tissue as

flowers differentiate (Halevy, 1985). These authors interpreted these variations in amino acid contents as metabolic signals of morphogenetic changes.

Few authors have studied the role of endogenous amino acids during somatic embryogenesis. Claporols et al. (1993) suggested that the accumulation of proline and alanine observed in maize calli on media containing proline, or glycine, favored embryogenic callus formation. In addition, modifying ammonium and nitrate concentration or adding amino acids to the culture medium improves somatic embryogenesis (Wetherell and Dougall, 1976; Eeuwens, 1976; Eeuwens, 1978; George and Sherrington, 1984; Mukhopadhyay and Desjardins, 1994; Paul et al., 1994). In coconut, higher ammonium absorption per gram of dry matter and an accumulation of proteins are seen during somatic embryogenesis initiation (Verdeil, 1993; Dussert et al., 1995).

In this study, a heterogeneity of endogenous amino acid composition is described in coconut calli on a somatic embryogenesis induction medium (SEIM). Also, changes in amino acid content of calli on an induction medium were compared with those of calli on a multiplication medium. This study was carried out from the initiation of embryogenesis up to the appearance of embryogenic cells and proembryos. Histological monitoring was carried out over this period.

Materials and Methods

Plant material

Inflorescences from the Marc Delorme Station (Abidjan, Côte-d'Ivoire) were sampled on 20 to 25-year-old hybrids (cultivar PB121: West African Tall × Malayan Yellow Dwarf hybrid, copyright CIRAND-CP).

Primary calli were obtained in the presence of 2,4-D and activated charcoal. After 6 months of culture, they appeared on the surface of the floral regions, and proliferated from a pseudocambial-like meristematic cell layer located at the callus edge and protected by a protoderm. Degeneration of the pseudocambial layer and reactivation of the protodermal layer were triggered by increasing either 2,4-D concentration in the media, or transfer frequency (from 60 days to 15 days) (Verdeil, 1993; Verdeil and Buffard-Morel, 1994). After proliferation of cells in the protodermal layer and after several subcultures, friable and homogeneous calli with high embryogenic potential were isolated, such as the L82 clone used in the present study (photo 1).

Each tube contained 200 mg of callus at the start of the experiment.

Culture media

The culture conditions were described by Verdeil et al. (1989). The media contained: Murashige and Skoog's macronutrients modified by Rabechault and Martin (KNO_3: 1200, KH_2PO_4: 700, NH_4NO_3 1300, $CaCl_2$, $2H_2O$ 360, $MgSO_4$, $7H_2O$ 300) (Murashige and Skoog, 1962; Rabechault and Martin, 1976), Nitsch's micronutrients (Nitsch, 1969), Morel and Wetmore's vitamins (1 mg L^{-1}) (Morel and Wetmore, 1951), EDTA iron (EDTA: 26 mg L^{-1}, $FeSO_4$, $7H_2O$: 24.9 mg L^{-1}), glucose (20 g L^{-1}), ascorbic acid (100 mg L^{-1}), myoinositol (100 mg L^{-1}), malic acid (100 mg L^{-1}), adenine sulfate (30 mg L^{-1}), biotin (0.005 mg L^{-1}); BAP (0.001 mg L^{-1}) and agar agar (7.5 g L^{-1}). The pH of the media was adjusted to 5 prior to autoclaving (110 °C, 20 min).

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

Experimental design

Two factors (treatment and time) were considered. Treatment involved three media: 1) a control medium (M100), and 2) two embryogenesis induction media (M100 with twice the concentration of macronutrients and higher 2,4-D concentration: 130 mg L^{-1} and 140 mg L^{-1} in the presence of 3 g L^{-1} of activated charcoal (medium M130 and M140, respectively).

Six sampling dates (T0, T8, T15, T28, T42, T60) were spread over a 60-day culture cycle. Each «medium × date» combination was replicated 3 times (3 tubes). All 54 tubes were placed in the culture room (temperature: 27 °C ± 1 °C, relative humidity: 55 % ± 1 %), according to a totally randomized design. Dry matter was estimated from a fraction of callus placed in a drying oven (48 h, 110 °C). The other fraction was used for amino acid titration.

Histological techniques

For each combination (medium × date), calli were monitored histologically using a technique described by Buffard-Morel et al. (1992). Tissues were fixed at least 1 day in a glutaraldehyde, paraformaldehyde and caffeine solution, then dehydrated in successive ethanol baths (30° to 100°). Transfer to an impregnation solution (LKB products) for at least 1 day preceded resin inclusion (LKB historesin). Sections (3.5 µm) were stained with a double P.A.S. stain (periodic acid/Schiff's reagent) for detection of carbohydrate compounds and naphthol blue black for detection of proteins.

Amino acid analysis

Calli were frozen in liquid nitrogen and stored at −30 °C. For analysis, fresh matter (200 mg) was crushed in liquid nitrogen, then in extraction mixture (chloroform and double distilled water (50/50), pH 3.9). The extraction product, kept overnight at 4 °C, was centrifuged at 4,500 rpm for 10 min. The aqueous phase was filtered successively through Millipore 0.45 µm and 0.2 µm filters, and stored at −30 °C.

The titration method involved amino acid derivation by phenylisothiocyanate (PTC) (Moulineau, 1994). For derivation, extracts (10 µL) were first evaporated (5 min) in the presence of 10 µL of a drying solution (water, ethanol, triethylamine; 2 : 2 : 1) and 10 µL of norleucine (221 mg mL^{-1}) used as internal standard. Then, 20 µL of derivation solution (ethanol, water, triethylamine and PTC; 7 : 1 : 1 : 1) was added. After 30 min, extracts were evaporated and kept at −30 °C.

PTC amino acids were separated by HPLC using a Picotag C18 250 × 4 mm column at 38 °C. The flow rate was 1 mL min^{-1}. Elution was carried out as follows: 1) two solvents: A (19 g L^{-1} of sodium acetate, 500 mg L^{-1} of triethylamine, pH 6.4) and B (acetonitrile, water; 6 : 4), 2) Elution gradient: 25 min from 10 % to 49 % solvent B, 3) A column rinsing gradient in solvent B (10 min) after each titration, and 4) Return to the initial conditions (i.e. 10 % B) in 10 min.

For HPLC, a known volume of eluant A was added to each evaporated sample. The PTC amino acids were detected in UV light at 254 nm. A standard amino acid solution (Pierce Chemical Co., Rockford, Illinois, USA) was used for calibration. Amino acid contents were expressed in mg g^{-1} for dry matter.

Statistical analysis

The 54 calli were considered as individuals and were characterized by their amino acid composition (variables). The data set forms an «individuals × variables» table.

Firstly, the clustering method was carried out on the 54 individuals in the experiment. The statistical distance chosen for estimating the difference between two individuals i and k was the Euclidian distance:

$$d_{ik} = \sum_{j=1}^{J} [(x_{ij} - x_{kj}) \cdot \sigma_j^{-1}]^2,$$

weighted for each variable j by the standard deviation s_j, where x_{ij} and x_{kj} are observed values of the variable j on individuals i and k, respectively.

Ward's aggregation criterion was adopted (Ward, 1963). The method results in a dendrogram, the interpretation of which defined groups of individuals. Hence, the calli were classified not according to the treatment they underwent, but according to their resemblance as regards their endogenous amino acid composition.

As the group definition was arbitrary, it was essential to ensure that results were biologically coherent. By a discriminant analysis (Romeder, 1973), the number of well-classified individuals per group was checked. This analysis allowed determination of which amino acid(s) led to the best between-group differentiation. One-way ANOVA (fixed model), followed by the Newman and Keuls test, (Newman, 1939; Keuls, 1952) was used to compare groups.

By means of this set of statistical methods, a callus typology was drawn up: I, II, etc. and it is thus possible to say, for example, that treatment X at time T gave x% type I calli, y% type II calli and z% type IV calli. Changes in the different types of calli can be studied over time.

Results

Characterization of different types of calli

Six amino acids were detected: alanine, leucine, proline, serine, threonine, and valine. Classification by the clustering method resulted in the dendrogram (Fig. 1). Five groups were defined:

1) group I with all the calli sampled at T0,
2) group II with the calli sampled at times T8 and T15 from the 2 embryogenesis induction media (SEIM),
3) group III with the calli sampled at times T8 and T15 from the multiplication medium,
4) group IV with the calli sampled at times T28, T42, and T60, either from the control medium or the SEIMs, and
5) group V which the calli sampled at times T28, T42, and T60 from SEIMs.

Description of callus types

Apart from the control date T0, two major phases can be highlighted:

1) an initial $\varphi 1$ phase (T8 and T15), corresponding to a latency phase as regards growth and adaptation to culturing, and
2) a second $\varphi 2$ phase (T28 to T60), corresponding to the growth phase.

Groups II and III belonged to the $\varphi 1$ phase, but corresponded to the two different types of medium: SEIM *vs* control. Endogenous amino acid contents revealed no difference between the two SEIMs. Discriminant analysis showed that the amino acid composition can be used to classify calli in groups II and III with no risk of error (100% well-classified individuals) (Table 2). Three amino acid contents were significantly lower on the SEIMs (Tables 1, 2, and 3):

1) valine, 10-fold lower,
2) proline, 1.5-fold lower, and
3) serine, 2-fold lower.

Discriminant analysis showed that valine was sufficient for callus sorting (Table 3).

Groups IV and V belonged to the $\varphi 2$ phase. Group V only contained calli from SEIMs, whereas group IV included calli

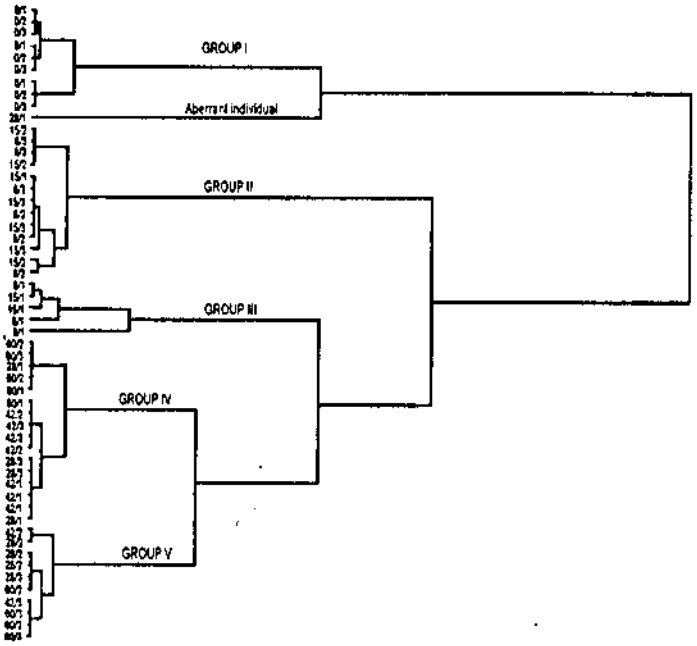


Fig. 1: Dendrogram representing callus distribution as a function of amino acid composition (Analysis carried out by the clustering method on the 54 individuals in the experiment) – Group I: TO – Group II: T8 and T15 on the two somatic embryogenesis induction media (SEIMs) – Group III: T8 and T15 on the control medium – Group IV: T15, T42, T60 on the three media – Group V: T15, T42, T60 on SEIMs – Aberrant individual: undetermined variability. 0, 8, 15, 28, 42 and 60 are the analysis times; 1, 2, 3 are the media: 1, multiplication medium; 2 and 3, SEIMs; * 60/2: individual wrongly classed in group IV but correctly classed in group V.

Table 1: Mean amino acid contents for the five groups of the clustering method.

Amino acids	group I	group II	group III	group IV	group V
valine	0.799	0.163	1.151	0.705	1.086
proline	0.257	0.103	0.151	0.152	0.221
serine	0.545	1.088	2.586	0.388	0.632
alanine	0.596	0.340	0.259	0.184	0.332
leucine	0.142	0.219	0.152	0.281	0.429
threonine	8.357	3.332	2.001	1.884	2.304
n[1]	9	13	5	16	10

[1] Number of individuals per group. The analysis involved 54 individuals. The 54th, an aberrant individual, was not taken into account. Results are expressed in mg per gram of dry matter.

from control and SEIMs. Calli of group V can be considered as orientated to somatic embryogenesis, and those of group IV as non-induced. SEIM gave two callus types with different amino acid compositions that seem to reflect their morphogenetic orientation.

These differences were confirmed by a histological study. On the control medium, callus growth and proliferation were ensured by the peripheral meristematic cell layer around a parenchymatous, sometimes degenerated, inner zone (Fig. 4). On SEIM, cells with a dense cytoplasm, a high nucleocytoplasmic ratio, a single large nucleolus, and characterized by the presence of starch and protein reserves appeared in the peripheral zone between the 15th and 28th day of cultures (Fig. 5). Cells were also surrounded by a thickened cell wall. After the 28th day of culture, these cells evolved into proembryos (Fig. 6), and embryogenic structures could be macroscopically observed on the friable and homogeneous calli (Fig. 3). This type of structure was observed for different coconut callus lines (Verdeil, 1993; Verdeil and Buffard-Morel, 1994; Verdeil et al., 1994) and confirmed the unicellular origin of somatic embryogenesis (Schwendiman et al., 1990; Michaux-Ferriere and Schwendiman, 1992). In contrast, some SEIM calli were still undifferentiated, and embryogenic cells and proembryos coexisted at a given date. Embryogenesis initiation is not synchronous, as deducted from amino acid composition and histological observation.

Discriminant analysis from amino acid content allowed classification of calli in groups IV and V with a 3.7 % risk of error (Table 2). Four amino acid contents were significantly higher on SEIM (Tables 1, 2, and 3):
1) proline and leucine with a 2-fold increased content, and
2) valine and alanine with a 1.5-fold increased content.
Proline was sufficient for callus sorting (Table 2).

A third discriminant analysis was carried out on callus groups II and V on SEIMs and belonging to the $\varphi 1$ and $\varphi 2$ phases, respectively. Amino acid composition could be used to classify the calli with no risk of error (100 % well-classified individuals) (Table 2). Three amino acid contents were significantly higher after T28 on SEIMs (Tables 1, 2, and 3):
1) valine with a 10-fold increased content, and
2) proline and leucine with a 2-fold increased content.
Valine was sufficient for callus sorting (Table 2).

Table 2: Discriminant analysis of groups 2 by 2:

Groups	II	III	IV	V	II	V
% correctly classed	100 %		96.3 %[1]		100 %	
Amino acid[2]	val		pro		val	
Mean[3]	0.163	1.151	0.152	0.221	0.163	1.086
Min.[4]	0.022	0.907	0.112	0.150	0.022	0.892
Max.[4]	0.263	1.311	0.186	0.276	0.263	1.295

Results are expressed in mg per gram of dry matter.
[1] One individual is wrongly classed in group IV but correctly classed in group V, (first individual 60/2 on the dendrogram in Fig. 1).
[2] Amino acid enabling a distinction to be made between calli. Pro = proline, Val = valine.
[3] Mean amino acid content within the groups (see Table 1).
[4] Minimum values (min.) and maximum values (max.) for proline or valine within the groups.

Table 3: Analysis of variance of amino acids contents in pairs of groups:

Groups	II and III			IV and V			II and V		
ANOVA	F	alpha[1]	Test[1]	F	alpha[1]	Test[1]	F	alpha[1]	Test[1]
val	255.37	0.000	***	22.48	0.01	**	471.27	0.000	***
pro	24.57	0.02	**	79.40	0.000	***	222.44	0.000	***
ser	11.54	0.04	**	2.14	15.58	NS	2.64	12.000	NS
ala	36.0	55.74	NS	49.95	0.000	***	0.000	94.85	NS
leu	3.54	7.96	NS	27.73	0.000	***	100.33	0.000	***
thr	3.11	9.83	NS	2.09	16.11	NS	3.25	8.65	NS

[1] Threshold < 5 %.
NS: test not significant at 5 %; ** and ***: degree of test significance.

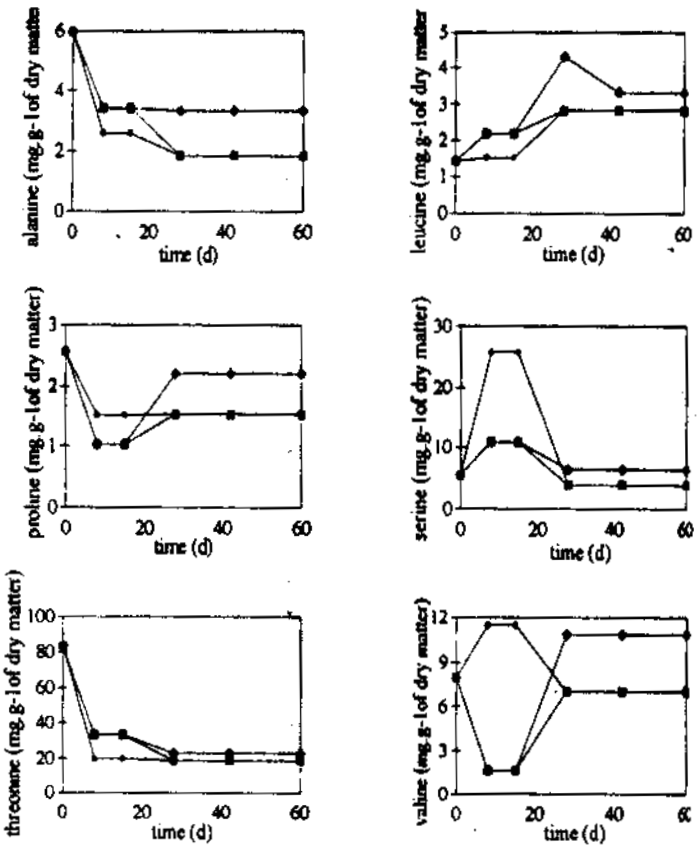


Fig. 2: Changes in mean amino acid content in the five CM groups as a function of time. Results are expressed in mg per gram of dry matter. d is the sampling time expressed in days. ● calli on the control medium, ■ calli on the SEIMs but not tending towards somatic embryogenesis, ◆ calli on the induction media tending well towards somatic embryogenesis.

Changes in amino acids over time

A graph of mean amino acid content over time was plotted for the five groups (Fig. 2). The kinetic study of amino acid contents emphasized the metabolic events characterizing embryogenesis initiation defined for calli on SEIMs.

Latency $\varphi 1$ phase

Inoculation led to a change in amino acid content irrespective of the type of medium. The content changed differently

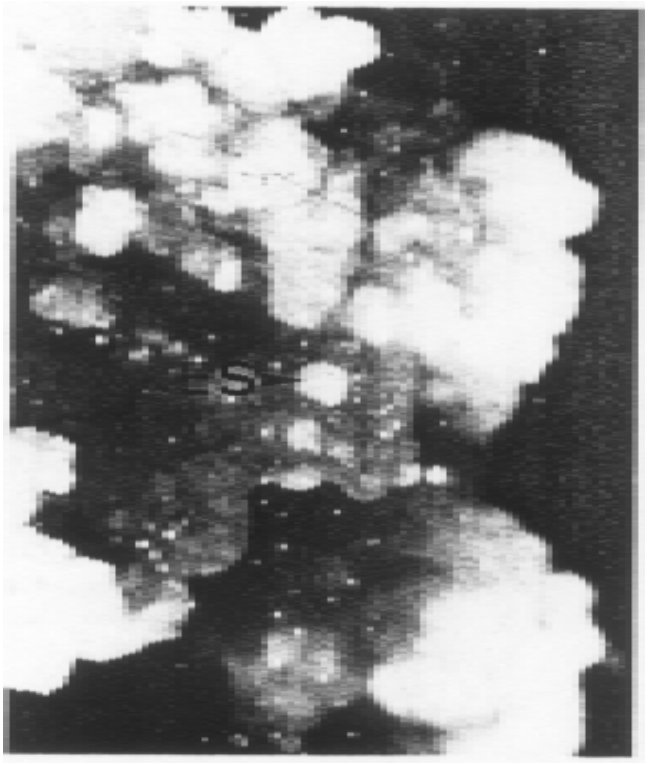


Fig. 3: Embryogenic callus of the line L82. Calli of this line are friable and homogeneous with a high embryogenesis potential. ES = embryogenic structures (× 4).

on SEIMs and control medium. Only the content of threonine, the main amino acid in the calli, was the same whatever the media and date.

Meristematic status on control medium was characterized by large increases in serine and valine contents, and a slight drop in proline content. During the $\varphi 1$ phase on SEIMs, somatic embryogenesis initiation was characterized by a marked decrease in proline and valine contents, and a slight increase in serine contents (Fig. 2). These modifications were concurrent with the appearance of embryogenic cells between the 8th and 15th day of culture (Figs. 5 and 6).

Growth phase $\varphi 2$

Calli on SEIMs revealed two tendencies during the $\varphi 2$ phase. Some calli were still at the meristematic stage. Their amino acid composition returned to that observed for control calli (Fig. 2). This change showed an adaptation to culture conditions.

The others tended towards somatic embryogenesis with a characteristic amino acid composition. Alanine content did not change compared with the $\varphi 1$ phase. Proline, valine and leucine contents increased concurrently with the appearance of proembryos between the 15th and 28th day.

Discussion

Heterogeneity of morphogenetic status of calli

Analysis of endogenous amino acid composition allowed the characterization of two callus types on SEIM. This typology has been associated with a histological confirmation. It is worth noting that heterogeneity was seen by Wetherell and Dougall (1976) in carrot calli on a solid medium during a mineral nutrition study; the variability in the physiological condition of the calli caused diversity in nitrogen compound translocation. In our approach, each callus has to be considered as a separate entity at a determined morphogenetic stage.

The first consequence was then the demonstration that only one amino acid (proline) is sufficient for sorting calli by their status. This possibility is of the greatest importance in the case of coconut, for which somatic embryogenesis is rare and time-consuming. This result for the L82 clone must be verified with other coconut callus clones before being generally inferred.

The second consequence of this study was demonstration of the inefficiency of classical ANOVA for comparison of treatments. Indeed, the mean of the «Induction Medium» treatment, i.e. the mean of the two amino acid profiles, has no biological significance. In contrast, we propose a statistical methodology based upon typology before comparison.

Changes in amino acid composition

Two different phases were observed considering the changes in amino acid composition.

The first was the latency $\varphi 1$ phase corresponding to a readaptation to the new culture conditions. This adaptation is characterized by a different use of metabolic resources between SEIM and control. On SEIM, the drop in valine, ser-

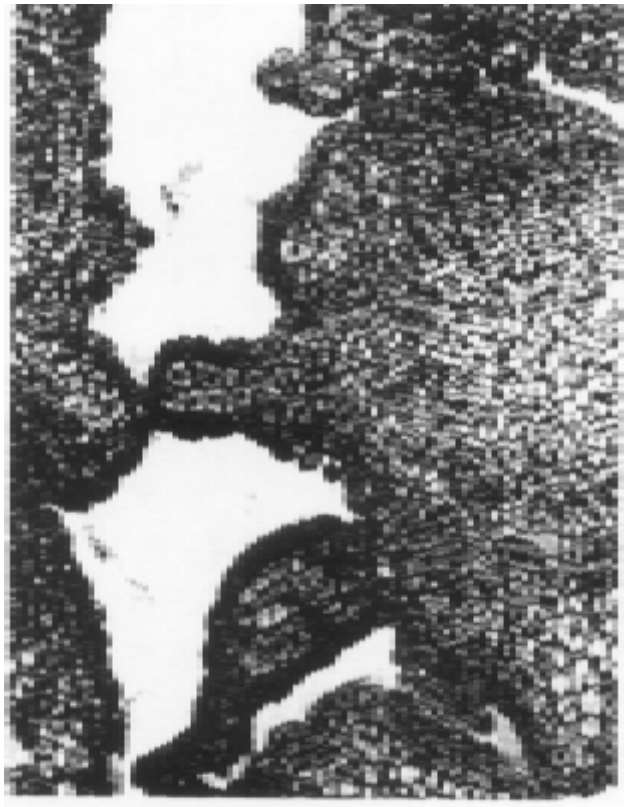
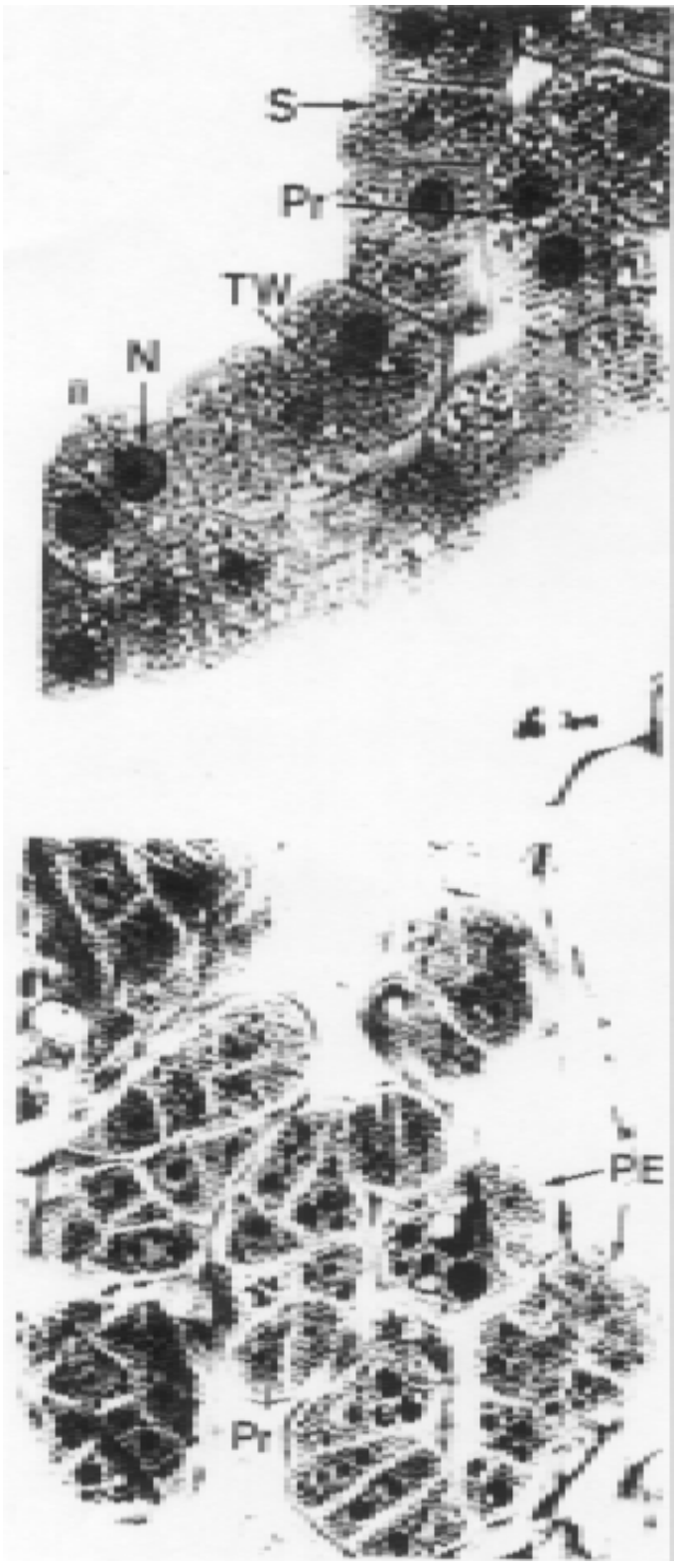


Fig. 4: Calli on the control medium. Callus growth and proliferation are ensured by a peripheral meristematic cell layer surrounding a parenchymatous inner zone (× 90).



Figs. 5, 6: Calli tending well towards somatic embryogenesis on the somatic embryogenesis induction media (SEIM). 5: Embryogenic cells with starch (S) and protein (Pr) reserves, formed between the 15th and the 28th day of culture. Embryogenesis is of unicellular origin. Single cells are surrounded by a thickened cell wall (TW). N = nucleus, n = nucleolus, (×800). 6: Proembryos (PE) formed after the 28th day of culture by segmenting division in a single embryogenic cell. Pr = protein reserves (×576).

ine, and proline contents was an initial reaction to the increases in 2,4-D concentration and mineral elements, since it enabled a distinction to be made between the calli on SEIM and calli maintained on control medium. However, it did not reveal any divergence between calli on SEIM. During this phase, histological study showed that embryogenic cells began to appear, but in small numbers. Embryogenesis was at too early a stage to reveal this divergence.

An HPLC study of mineral nutrient contents in the media over time revealed greater ammonium absorption on SEIM (Dussert et al., 1995). The higher endogenous nitrogen content was therefore in contradiction with the drop in the three amino acids. This drop could be due to the use of these compounds rather than to a decrease in their synthesis. The drop in valine content was the main signal of changes in the media. Valine has primarily been described as being a reduced nitrogen source in the culture media, but its presence is often critical for callus or cell growth (George and Sherrington, 1984). The change in serine content was a very early metabolic signal. In fact, its content characterized the calli on SEIM. It ceased to be so in the growth $\varphi 2$ phase. It is with noting that serine is primarily described as being a precursor of tryptophan during endogenous auxin synthesis (Wareing and Philips, 1973; Richter, 1993). The drop in proline content was also a metabolic signal during the $\varphi 1$ phase. On SEIM, increasing the 2,4-D concentration and doubling the mineral macroelement concentration cause a stress on tissue in culture. Such a stress is often necessary for somatic embryogenesis initiation for other plants (Carman, 1990). Stress is known to induce accumulation of proline (Handa et al., 1986; Pulich, 1986; Pesci et al., 1992; Dumet, 1994; Moulineau, 1994). In our study, a drop in proline content is then at variance with the expectations.

During the second phase (growth $\varphi 2$ phase), the proline content increase (Fig. 2) was the most important signal of somatic embryogenesis initiation. This has already been observed during embryogenesis acquisition in maize calli and might protect calli against metabolic changes due to *in vitro* culture and somatic embryogenesis (Claporols et al., 1993). Nevertheless, this interpretation does not explain the drop observed during the $\varphi 1$ phase, the more stressful phase. Accumulation may correspond to a physiological feature of orientation towards somatic embryogenesis.

This different trends in proline, valine, leucine and alanine contents in calli towards somatic embryogenesis could simply be indicative of a dominant need for protein synthesis. This is confirmed by the accumulation of storage proteins (Figs. 5, 6), and an increase in protein synthesis has been noted between the 15th and 28th day of culture under embryogenesis conditions in coconut (Dussert et al., 1994). It is also interesting to note that during the zygotic embryogenesis of other species, the storage proteins in seeds contain significant quantities of these four amino acids (Mossé and Baudet, 1983).

Conclusions

Amino acids appear to be valid quantitative markers of calli well orientated towards embryogenesis in the L82 coconut clone. By extending our approach to other clones, it

would be easier to screen the most embryogenic lines of coconut. In addition, we wish to determine the precise role of these amino acids and of their metabolism, especially by using labeled nitrogen and by monitoring their incorporation into calli. The study of nitrogen metabolism may allow us to optimize culture conditions by acting on the nitrogen source and on its concentration in media (modification of the nitrogen concentration, addition of amino acids), there by ameliorating conditions of embryogenesis initiation.

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