The zygotic embryo: a model for physiological studies in coconut

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1. Introduction

Due to the large weight and size and lack of dormancy of coconut (*Cocos nucifera* L.) seeds, *in vitro* culture of zygotic embryos provides a useful alternative for collecting and exchanging germplasm (Assy-Bah *et al.*, 1987). Sampling methodology and *in vitro* culture protocols for coconut zygotic embryos have been well documented (Assy-Bah, 1986; Rillo and Paloma, 1992; Ashburner *et al.*, 1994; Sigurma *et al.*, 1994; Rival *et al.*, 1996). Nevertheless, when compared to seedlings, *in vitro* grown coconut plantlets show a slower development in the nursery after acclimatisation (Assy Bah *et al.*, 1989). Thus, the intrinsic quality of *in vitro* grown coconut plantlets needs to be improved. Furthermore, zygotic embryo culture provides a model system that could be applied to improve conditions used for the *in vitro* development of somatic embryos (Buffard-Morel *et al.*, 1992; Verdeil *et al.*, 1992).

During the transfer to *ex vitro* conditions, the physiological status of *in vitro* grown plantlets is an important factor determining success rates (Debergh, 1991; Van Huylenbroeck and Debergh, 1996). In order to optimise this very critical phase, apart from the control of water stress (Santamaría and Davies, 1994), investigations on the photosynthetic ability of plantlets during the *in vitro* process are necessary.

The coconut palm, like many *in vitro* grown C_3 species, can assimilate inorganic carbon *via* two independent carboxylation pathways. One occurs in the chloroplasts through the action of Ribulose 1,5-bisphosphate carboxylate /

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oxygenase (RubisCO, EC:4.1.1.39). The activity of this enzyme is closely linked to the development of chloroplasts and reflects the integrity of the photosynthetic apparatus. The other pathway takes place in the cytosol through the Phosphoenolpyruvate carboxylase pathway (PEPC, EC:4.1.1.31) (Nato and Vidal, 1983; Neuman *et al.*, 1989; Lavergne *et al.*, 1992; Hdider and Desjardins, 1994; Rival *et al.*, 1996; 1997a). PEPC is an ubiquitous, highly regulated enzyme in plants (Chollet *et al.*, 1996). The C_3-PEPC feeds carbon into the tricarboxylic acid cycle to provide precursors for various biosynthetic processes, including amino acid biosynthesis (Huber and Kaiser, 1996). Thus the ratio of PEPC:RubisCO activities could be a reliable indicator of the relative conditions of non-photosynthetic (heterotrophic) and photosynthetic (autotrophic) pathways of CO_2 fixation (Kumar *et al.*, 1988). Nevertheless, carboxylase activities, as measured *in vitro* on protein extracts, can only give an estimation of the optimal capacity for CO_2 fixation in the plant material. These enzymatic studies need to be complemented with investigations on *in planta* photosynthetic parameters. The measurement of fluorescence emission from the chlorophylls of the photosynthetic systems provides a non-invasive approach to study the photochemical events of photosynthesis and provides accurate information on the activity of the photosynthetic apparatus through the efficiency of photosystem II (PSII) (Kraus and Weis, 1991; Baker, 1993). This technique has already been successfully applied to *in vitro* grown plant material in order to assess *in vitro* photosynthesis (Capellades *et al.*, 1989; Capellades *et al.*, 1990a; Pospisilova *et al.*, 1993; Hdider and Desjardins, 1994; Rival *et al.*, 1997a).

The photosynthetic ability of plantlets needs to be confirmed by CO_2-exchange measurements *in planta*. This approach has been developed for the *in vitro* culture of various plant species such as strawberry, potato, tobacco and rose (Capellades *et al.*, 1990b; Kozai *et al.*, 1991; Pospisilova *et al.*, 1992; 1993; Van Huylenbroeck and Debergh, 1996). To date, in coconut, the photosynthetic characteristics of *in vitro* grown plantlets have not been extensively studied.

The purpose of the work reported here was to investigate the photosynthetic status of *in vitro* grown coconut plantlets obtained by zygotic embryo culture, combining various complementary approaches applied both *in vitro* and *in planta*. Also this paper points out the importance of undertaking studies involving the zygotic embryo model as a mean to achieve in the long term, efficient somatic embryogenesis and reliable clonal plantlet production in coconut. Results of studies on oil palm (*Elaies guinensis*) obtained in our group, are presented here to show the potential of biochemical markers for embryo maturation.

2. Studies on *in vitro* photosynthesis

2.1. Materials and methods

Plant material.- Zygotic embryos of the Malayan Yellow Dwarf (MYD) coconut (*Cocos nucifera* L.) ecotype were collected in Indonesia, México and Côte d'Ivoire. The MYD ecotype was chosen because it exhibits autogamous reproduction; thus heterogeneity between zygotic embryos would be minimised allowing in turn for more satisfactory results in embryo culture (Assy Bah *et al.*, 1986; 1987). As a control, we used an acclimatised six year-old autotrophic coconut palm that was grown in a computer monitored tropical glasshouse with a temperature of 27±2°C, a relative humidity of 70 ± 5 %, and natural sunlight (PAR=150-250 µmol m^{-2} s^{-1} at the plantlet level).

In vitro culture conditions.- The tissue culture medium was composed of Murashige and Skoog (1962) micro- and macro-elements modified by Rabéchault and Martin (1976), vitamins according to Morel and Wetmore (1951), 60 g l^{-1} sucrose and 2 g l^{-1} activated charcoal, according to the protocol described by Assy-Bah *et al.* (1989). The pH was adjusted to 5.0 before adding charcoal, then the medium was autoclaved (110°C, 103 Kpa, 20 min). Zygotic embryos were collected and cultured as previously described (Assy Bah *et al.*, 1987), except that liquid medium was used throughout the *in vitro* culture protocol (Rival *et al.*, 1996). Excised coconut embryos were grown in Pyrex tubes in the dark (T°: 27 ± 1°C). They were transferred every two months onto 20 ml of fresh liquid medium. As soon as the first leaf and a complete root system were fully developed, plantlets were transferred onto 100 ml. liquid medium in one-litre culture bottles under light (45 ± 5 µmol m^{-2} s^{-1}). Plantlets were acclimatised when they were 6 months old and displayed 2 to 3 unfolded green leaves.

Physiological parameters.- The various methods employed for the estimation of chlorophyll fluorescence, the measurements of CO_2 exchange, transpiration rates and the estimation of chlorophyll concentrations have been previously described (Triques *et al.*, 1997a; Triques *et al.*, 1997b). Enzyme extraction and measurements of PEPC and RubisCO capacities were performed according to Rival *et al.* (1996). RubisCO was quantified in protein crude extracts by rocket immunoelectrophoresis, according to the method of Laurell (1966), as modified by Lavergne *et al.* (1992).

2.2. Results and discussion

Chlorophyll fluorescence.- In the dark-adapted leaf, an index for the maximal quantum yield of photochemistry through PSII ($\varnothing_P^{MAX}$) was calculated as (Fm-F0)/Fm (Kitajima and Butler 1975). The actual quantum yield of PSII photochemistry in light-adapted leaves ($\varnothing_P$) was calculated as (F'm-Fs)/F'm (Havaux *et al.*, 1991). Chlorophyll fluorescence parameters ($\varnothing_P$ and $\varnothing_P^{MAX}$) were measured in dark-grown leaves and during greening under PAR (Table 1). $\varnothing_P^{MAX}$ was very low in dark-grown plantlets. This parameter increased during the greening of leaves as early as 2 weeks after cultivation under PAR. Values for $\varnothing_P$ and $\varnothing_P^{MAX}$ were not significantly different either in *in vitro* grown plantlets (after 4 weeks under PAR) or in the acclimatised coconut palm.

Net photosynthesis and transpiration.- Net photosynthesis rates were measured through CO_2 exchange in leaves from *in vitro* grown plantlets (Table 2). During the greening of leaves, the net CO_2 exchange increased. The photosynthetic rate in *in vitro*-cultured plantlets was then half of that measured in the autotrophic coconut palm. The transpiration rate in *in vitro* dark-grown plantlets was very low (0.04 mmol H_2O m^{-2} s^{-1}). During the greening of leaves, transpiration increased up to a value that was not significantly different from the rate measured in an autotrophic palm.

Table 1. Changes in the maximal quantum yield ($\varnothing_P^{MAX}$) of PSII photochemistry (in dark-adapted leaves) and the actual quantum yield ($\varnothing_P$) of PSII photochemistry (in light-adapted leaves) in coconut leaves sampled at various stages of *in vitro* development. Reported values are the means of 3 independent measurements ± standard deviation (SD). Results of one way analysis of variance (ANOVA) are given: Snedecor's variable (F) and Type I Error (p).

Culture stage	$\varnothing_P^{MAX}$ (Fm-F0)/Fm	$\varnothing_P$ (F'm-Fs)/F'm
Dark-grown plantlet	0.29 ± 0.02^a	ND
1 week PAR	0.58 ± 0.02^a	0.33 ± 0.03^a
2 weeks PAR	0.71 ± 0.05^c	0.41 ± 0.09^b
4 weeks PAR	0.72 ± 0.04^c	0.45 ± 0.03^b
Autotrophic plant (reference)	0.76 ± 0.01^c	0.50 ± 0.03^b
ANOVA		
F	138.67	6.94
p	0.0000	0.0033

Table 2. Changes in the net photosynthesis estimated through CO_2 exchanges and transpiration rates during the *in vitro* development of coconut zygotic embryos into plantlets. Results of one way analysis of variance (ANOVA) are given: Snedecor's variable (F) and Type I Error (p).

Culture stage	Photosynthetic rate $\mu mol\ CO_2\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ H_2O\ m^{-2}\ s^{-1}$
Dark-grown plantlet	-0.500^{a}	0.040^{a}
1 week PAR	0.271^{ab}	0.340^{a}
2 weeks PAR	0.940^{b}	1.135^{b}
4 weeks PAR	1.144^{b}	1.140^{b}
autotrophic plant (reference)	2.430^{c}	1.137^{b}
ANOVA		
F	11.40	9.458
p	0.0006	0.0027

Table 3: Changes in chlorophyll content and Chl a/Chl b ratio, during the *in vitro* development of coconut zygotic embryos into plantlets. Reported values are the means of 3 independent measurements ± SD. Results of one way analysis of variance (ANOVA) are given: Snedecor's variable (F) and Type I Error (p).

Culture stage	Total chlorophyll (a + b) $mg\ g^{-1}\ FW$	Chl a / Chl b ratio
Mature embryo	0.005 ± 0.000^{a}	0.625 ± 0.739^{a}
Dark-grown plantlet	0.071 ± 0.080^{a}	0.678 ± 0.080^{a}
1 week PAR	0.134 ± 0.106^{a}	3.695 ± 0.313^{b}
2 weeks PAR	0.456 ± 0.035^{a}	3.249 ± 0.568^{b}
4 weeks PAR	0.329 ± 0.190^{a}	2.600 ± 0.705^{b}
6 weeks PAR*	0.921 ± 0.228^{b}	3.661 ± 0.227^{b}
Autotrophic plant (reference)	1.451 ± 0.277^{b}	3.144 ± 0.092^{b}
ANOVA		
F	30.81	35.97
p	0.0000	0.0000

* Ready for acclimatisation.

Table 4. Changes in total soluble protein contents and RubisCO and PEPC specific capacities during the *in vitro* development of coconut zygotic embryos into plantlets. Reported values are the means of three independent measurements ± SD.

Culture stage	TSP content mg g^{-1} FW	PEPC capacity µmol CO_2 h^{-1} mg^{-1} TSP	RubisCO capacity µmol CO_2 h^{-1} mg^{-1} TSP	PEPC:RubisCO ratio
Mature embryo	14.40 ± 1.71^{b}	17.83 ± 0.51^{a}	0.20 ± 0.00^{a}	89.17 ± 2.56^{a}
Dark grown plantlet	0.94 ± 0.00^{a}	40.30 ± 11.31^{a}	0.95 ± 0.21^{ab}	42.14 ± 2.49^{a}
1 week PAR	3.07 ± 0.49^{a}	4.17 ± 2.55^{b}	2.12 ± 1.02^{bc}	2.31 ± 1.44^{b}
2 weeks PAR	5.18 ± 0.25^{a}	3.14 ± 0.79^{b}	5.41 ± 1.19^{d}	0.61 ± 0.24^{b}
4 weeks PAR	5.13 ± 1.09^{a}	1.38 ± 1.00^{b}	3.83 ± 0.41^{c}	0.36 ± 0.26^{b}
6 weeks PAR*	20.56 ± 3.38^{c}	0.08 ± 0.03^{b}	2.83 ± 0.34^{bc}	0.03 ± 0.01^{b}
Autotrophic plant	14.26 ± 5.09^{b}	0.33 ± 0.36^{b}	6.60 ± 1.56^{d}	0.04 ± 0.04^{b}
ANOVA				
F	49.47	19.40	23.22	121.34
p	0.0000	0.0000	0.0000	0.0000

* Ready for acclimatisation.

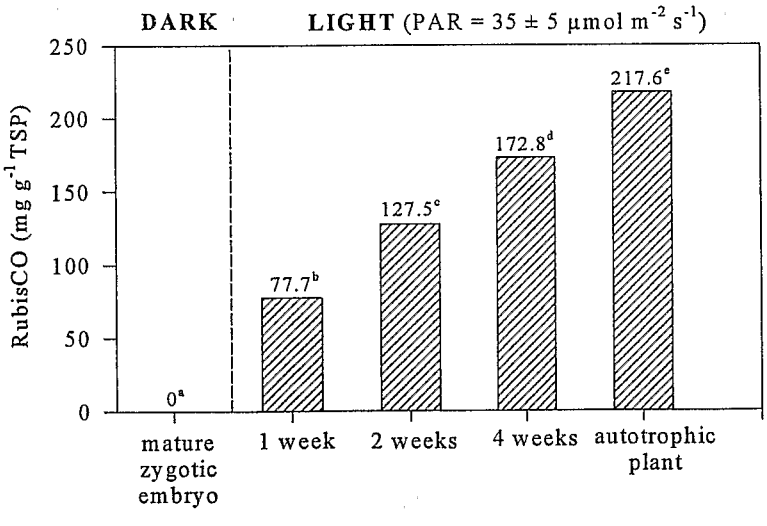

Figure 1. Changes in the RubisCO content (determined by immunoelectrophoresis), during the *in vitro* development of coconut zygotic embryos into plantlets and that of an autotrophic plant as a reference.

Chlorophyll content.- During the development of zygotic embryos in the dark, very low chlorophyll (a and b) levels were found, as expected (Table 3). After exposure to light, the total chlorophyll content increased up to 0.921 $mg.g^{-1}$ FW in green plantlets just before acclimatisation (i.e. 6 weeks under PAR). The chlorophyll content of the autotrophic palm was significantly higher. Chl a:Chl b ratios were low at the beginning of the culture process, when plantlets were cultivated in the dark. As of one week under PAR, this ratio was found to be not significantly different from that of *in vitro* grown plantlets, as compared with that measured for an autotrophic palm.

RubisCO and PEPC capacities and PEPC:RubisCO ratios.- Carboxylase (PEPC and RubisCO) capacities were measured in mature zygotic embryos, etiolated leaves and greening leaves (Table 4). In the mature embryo, the total soluble protein (TSP) content was high, while in greening leaves lower TSP contents were measured. A significant increase was noted after 6 weeks under light. During the *in vitro* culture process, the PEPC capacity of leaves drastically decreased. In contrast, the RubisCO capacity increased throughout the *in vitro* culture period. Consequently, the PEPC:RubisCO ratio dropped from 89.17 in the mature embryo down to 0.03 in ready-to-acclimatise plantlets (6 weeks under PAR), a ratio similar to that found in the autotrophic coconut palm.

Furthermore, we have demonstrated (with a different batch of plantlets) that *in vitro* grown coconut plantlets during acclimatisation showed a faster decrease in their PEPC:RubisCO ratio than seedlings, suggesting that an earlier transition from a heterotrophic to an autotrophic mode of carbon fixation takes place in the *in vitro*-derived material (Triques *et al.*, 1997a). Just before acclimatisation, the RubisCO activity in *in vitro*-derived plantlets was lower than that in seedlings of the same age. Nevertheless, after acclimatisation, RubisCO activities were comparable in both *in vitro* and *in planta* germinated material.

Quantification of RubisCO.- RubisCO was quantified in mature embryos, dark grown plantlets and in greening leaves (Fig. 1). RubisCO content increased from 0 $mg.g^{-1}$TSP in dark-grown leaves to 172.8 $mg.g^{-1}$TSP in leaves after 4 weeks under PAR. The RubisCO content was found to be 217.6 $mg.g^{-1}$TSP in the autotrophic coconut palm.

The present results demonstrate the establishment of photosynthetic metabolism during the *in vitro* development of coconut plantlets. Several notable similarities have been observed between *in vitro* grown coconut plantlets and the adult autotrophic coconut palm. The data suggest that there is a high level of PSII

activity in the vitroplant. Our data are consistent with the $\varnothing_P^{MAX}$ values obtained for oil palm using both *in vitro* grown (0.74) and acclimatised plantlets (0.79) (Rival *et al.*, 1997b) or measured in several species cultivated *in vitro*, such as tobacco (0.82) or potato (0.73) (Pospisilova *et al.*, 1993). $\varnothing_P$ is a reliable index of quantum yield of PSII photochemistry in illuminated leaves (Genty *et al.*, 1989) and reflects, for coconut, a fully functional linear electron transport chain in *in vitro* grown plantlets.

Both photosynthesis and chlorophyll fluorescence were found to increase concomitantly during the *in vitro* culture process, suggesting an increase in CO_2 assimilation. The existence of a correlation between $\varnothing_P$ and CO_2 fixation measurements under non-photorespiratory conditions has been previously reported Genty *et al.*, 1989; Krause and Weis, 1991). Nevertheless, the photosynthetic rate measured in *in vitro* grown plantlets remained half as much as that of the autotrophic palm. Generally, higher photosynthetic rates are recorded in seed-derived plants as compared with *in vitro* grown material (Pospisilova *et al.*, 1992). The photosynthetic rate obtained for the adult autotrophic coconut palm that was grown in a tropical glasshouse was 2.43 µmol $CO_2.m^{-2}s^{-1}$. It must be noted that this value was much lower than the one measured with the same ecotype cultivated in natural tropical conditions (11 ± 5 µmol CO_2 $m^{-2}s^{-1}$) and therefore under a markedly different light and temperature regime (Reppelin *et al.*, 1997). Many authors reported that unacclimatised leaves of *in vitro* cultured plantlets showed permanent stomatal opening or poor control of water loss (Drew *et al.*, 1992; Santamaría and Davies, 1994). The observation that transpiration rates are similar in *in vitro*-cultured plantlets and in the autotrophic palm suggests that stomatal opening is correctly regulated in *in vitro* coconut plantlets. Nevertheless, this feature must be assessed more precisely, using field-grown palms in natural tropical conditions as a standard.

The low photosynthetic rate measured in *in vitro* grown coconut plantlets was accompanied by a lower chlorophyll content as compared with the autotrophic palm. The chlorophyll content measured for *in vitro* grown coconut plantlets was of the same order as that determined for example, in tobacco *in vitro* grown plantlets (1.09 mg g^{-1}fw) (Pospisilova *et al.*, 1993). The Chl a : Chl b ratio measured in *in vitro* grown coconut plantlets was comparable to that measured in vitroplants from other species (Lichtentaller *et al.*, 1981; Serret *et al.*, 1996).

At the early stages of *in vitro* culture (*i.e.* 1 week under PAR) the PEPC:RubisCO ratio was very high, due to a high PEPC capacity. High PEPC capacities (6.6 µmol CO_2 h^{-1} mg^{-1} TSP) were also measured in shoot-forming cotyledons of *Pinus*

radiata (Kumar *et al.*, 1988) and in young somatic embryos of oil palm (5.2 µmol CO_2 h^{-1} mg^{-1} TSP) (Rival *et al.*, 1997b). The occurrence of a transient preferential CO_2 fixation through PEPC when C_3 plants or isolated cells are cultivated *in vitro* has been described in several species (Nato *et al.*, 1981; Neuman *et al.*, 1989; Hdider and Desjardins 1994; Rival *et al.*, 1997b). The PEPC:RubisCO ratio decreased in *in vitro* grown plantlets down to 0.03, a value similar to the one measured in autotrophic coconut palm. Similar patterns were observed in *in vitro* grown plantlets of oil palm (Rival *et al.*, 1996), in which a depletion of the PEPC:RubisCO ratio (down to 0.06) was noted during the *in vitro* development of somatic embryos. During *in vitro* growth and development, coconut plantlets showed a transition from a heterotrophic to an autotrophic (RubisCO-mediated) mode of carbon fixation. Indeed, a marked decrease in PEPC, concomitant with substantial increase in RubisCO capacity, was observed. In the case of coconut plantlets grown *in vitro*, the RubisCO capacity and content were lower than in the adult autotrophic coconut palm and this could explain the low rates of CO_2 assimilation found in *in vitro* grown plantlets. The high level of sucrose present in the culture medium (60 g l^{-1}) could have affected the RubisCO capacity. Indeed, exogenous carbohydrates have been reported to induce a depletion in RubisCO efficiency (Neuman *et al.*, 1989; Hdider and Desjardins, 1994; Van Huylenbroeck and Debergh, 1996) and photosynthetic rate (Serret *et al.*, 1996). A reduction of the sucrose level in culture medium at the end of the *in vitro* process could therefore allow an increase in photosynthesis, probably *via* an increase in RubisCO efficiency (see Santamaría *et al*, this volume).

In vitro-cultured coconut plantlets displayed an early initiation of a photosynthetic metabolism. Concomitant changes in several parameters (Φ_P^{MAX}, Φ_P, CO_2 fixation, PEPC:RubisCO ratio and transpiration rates) were measured. However, a lower rate of net photosynthesis was recorded in *in vitro* grown plantlets as compared with the acclimatised palm. This could be explained by lower RubisCO content and activity, together with a lower chlorophyll content compared to those found in the acclimatised palms. This work now needs to be complemented by the monitoring of the parameters studied here as a whole, during the subsequent stage of acclimatisation of plantlets.

3. Biochemical markers for embryo maturation

By studying the zygotic embryo development, it has been possible to identify markers for zygotic embryo maturation, namely storage proteins and oligossacharides. These markers may be useful in studies aimed to promote

somatic embryo maturation and their ability to withstand desiccation. This could be very important for mid term storage of isolated somatic embryos as those obtained from suspension cultures for large-scale propagation of improved planting material.

3.1. Storage proteins

Redenbaugh *et al.* (1986) have suggested that storage proteins could be relevant markers in the assessment of the maturation of somatic embryos and hence of the quality of the resulting plantlets. Plant regeneration protocols have been improved through the characterisation of the storage proteins and the control of their synthesis during the maturation of somatic embryos in numerous species (Roberts *et al.*, 1990; Misra 1994; Xu and Bewlley 1995; Mc Kersie *et al.*, 1995).

There is little information available about seed storage proteins of palm species. Ultrastructural studies have revealed protein crystalloids in the protein bodies of endosperm cells from Washingtonia (*Washingtonia filifera* Wendl.) and coconut palms (Chandra-Sekhar and De Mason, 1988; De Mason and Chandra-Sekhar, 1990). Sjogren and Spychalski (1930) have noted in the endosperm of coconut palm a salt-soluble protein termed "cocosin", which has been characterised as an 11S globulin (Carr *et al.*, 1990). In addition, polypeptides in total protein extracts have been shown to be recognised by antibodies to 7S and 11S soybean globulins (De Mason and Chandra-Sekhar, 1990).

Research work has been recently performed in our group (Morcillo *et al.*, 1997a; Morcillo *et al.*, 1997b; Morcillo *et al.*, 1999) on storage proteins in oil palm. Storage proteins that accumulated during oil palm embryo development were extracted, purified and characterised. Only water- and low-salt-soluble proteins, with respective sedimentation coefficients of 2S and 7S, were detected in mature embryos. After purification by gel filtration, the various protein classes identified were characterised by electrophoresis and amino acid composition analysis. The 2S proteins comprise polypeptides of 22 kD and 19 kD, which are acidic (pI<6) and basic (pI>9) respectively. The 7S proteins predominate and are heterogeneous oligomers (MW 156 kD and 201 kD), comprising a polypeptide triplet of Mr between 45 and 65 kD with no disulphide bonds. Their amino acid composition is broadly similar to those of the 7S proteins of other monocotyledon embryos, but differs from those of the legume 7S vicilins. Histological examinations and electrophoresis showed that the 2S and 7S proteins appeared at the third month after fertilisation, and no qualitative changes were detected up to the sixth month of embryo development.

Merkle *et al.* (1995) proposed storage proteins as markers for embryo maturation. The 7S globulins, which predominate both in oil palm embryos and in many other monocotyledon embryos might therefore potentially serve as maturation markers in the study of somatic embryogenesis. Using Western blotting and ELISA tests, 7S globulins have recently been detected and quantified in somatic embryos derived from embryogenic suspensions (Morcillo *et al.*, 1997b, Morcillo *et al.*, 1999). Preliminary studies performed in our group using the Western blotting technique have revealed that anti-7S globulin polyclonal antibodies could clearly detect these proteins in coconut mature zygotic embryo. This result opens the possibility to use storage proteins as markers of somatic embryo maturation in coconut.

3.2. Oligosaccharides

Oligosaccharides were reported to play a role in the protection of cytoplasm and membranes during seed desiccation (Koster and Leopold, 1988). Raffinose and stachyose could prevent sucrose crystallisation during dehydration, hence allowing the occurrence of a glassy state and preventing crystallisation damages (Koster 1991). The [sucrose : (raffinose + stachyose)] ratio thus may be considered to be a reliable indicator of the capacity of embryos to withstand desiccation.

Accordingly, an study was carried out in our laboratory to evaluate changes in these indicator oligosaccharides during the *in vitro* development of the oil palm zygotic embryos. Sugars were extracted in 80% alcoholic solution and analyzed using a ion exchange-HPLC (DIONEX) using a NaOH gradient (0-0.2 mM, with a flow of 1 ml min^{-1}), according to Aberlenc-Bertossi *et al.* (1995a). This study showed that the development of the oil palm zygotic embryo is characterised by the accumulation of non-reducing sugars and the depletion of reducing sugars content. The [sucrose: (raffinose + stachyose)] ratio was found to decrease dramatically from 68 down to 14 between the 3rd and the 4th month after fertilisation and to drop to 5.2 at the 6th month (Aberlenc-Bertossi *et al.*, 1995a; Aberlenc-Bertossi *et al.*, 1995b). The role of oligosaccharides will be further investigated throughout the zygotic embryo development in relation to the acquisition of tolerance to desiccation. Such studies will find short-term applications in the storage of desiccated somatic embryos, thus opening the way to the concept of 'artificial seeds' in oil palm.

4. Conclusion

The coconut zygotic embryo is an ideal choice of material for the collection and transfer of germplasm, as has already been well documented. Furthermore, it also

constitutes a valuable model for physiological studies aimed at investigating various aspects of primary metabolism in *in vitro* grown plantlets. The ultimate aim of such studies is the application of results obtained with zygotic embryos to in vitro grown plant material obtained by somatic embryogenesis. The results presented here on the development of photosynthetic capacity and markers for embryo maturity, open the way for new fields of research in coconut *in vitro* physiology.

Given the difficulties encountered for the implementation of regeneration protocols *via* somatic embryogenesis for this recalcitrant species, attention must now be paid to in-depth studies of carbon and nitrogen metabolism in the developing zygotic embryo. The latter will serve as a reference towards understanding the growth and development of somatic embryos.

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