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ORIGINAL PAPER

In vitro conservation of oil palm somatic embryos for 20 years on a hormone-free culture medium: characteristics of the embryogenic cultures, derived plantlets and adult palms

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Abstract This study was conducted over a period of 20 years, to assess the problems involved in developing subcultures over a very long period, of oil palm (*Elaeis guineensis* Jacq.) somatic embryos which were maintained in vitro on a Murashige and Skoog mineral-based culture medium, without growth regulators. Analysis of the proliferation rate of the embryogenic cultures, along with the survivability of the regenerated plantlets after their transfer into soil and of the flowering of the derived adult palms has been conducted for cultures maintained in vitro during 1 to 20 years. From the ninth year of maintenance, the tissue quality of the somatic embryos gradually began to decline. However, after more than 20 years, 30% of the 20 clones tested still continued to proliferate satisfactorily on the same maintenance medium, keeping their multiplication potential intact. Even though a depressive effect of the age of the lines has been observed on the survival capacity of plants under natural conditions, it is noteworthy that among the clones originating from 20-year-old cultures only eight of them (40%) have exhibited the "mantled" floral abnormality. Different hypotheses concerning the origin of the disruptions observed on the in vitro cultures, plantlets and adult palms that occur over a very long period of in vitro conservation are discussed.

Keywords In vitro conservation · Oil palm · Somatic embryogenesis · Somaclonal variation · "Mantled" floral abnormality

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
NAA	α-Naphthalenacetic acid
IAA	Indoleacetic acid
MS	Murashige and Skoog medium
FGC	Fast-growing calli
NC	Nodular calli

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is classed today as the top oil crop, ahead of soybean (FAS-USDA 2008). In order

to vegetatively multiply the best hybrid genotypes of this perennial cross-fertilising species of economic interest, several methods for in vitro regeneration through somatic embryogenesis on callus have been developed (for review: Rival and Parveez 2004). Starting from the protocol published by Pannetier et al. (1981), we developed a strategy for industrial in vitro propagation of plants using proliferating lines of somatic embryos (Duval et al. 1988; Konan et al. 2006a).

By applying the improved cryopreservation protocol of Dumet et al. 1993, we also succeeded in conserving clumps of somatic embryos of our best clones in liquid nitrogen at −196°C. The positive effects of this strategy have been reported for the conservation of oil palm clonal resources, especially the vigorous development in the field of plants derived from cryopreservation, compared to material undergoing many successive subcultures (Konan et al. 2007a). At the same time, lines of somatic embryos have been maintained in vitro by regular subculturing for continuous proliferation in vitro (Durand-Gasselin et al. 1993). This kind of conservation made them usable at any time for plant production. However, the drawback of in vitro conservation remains the high cost of upkeep and the risk of losses through microbial contamination (Reed and Tanprasert 1995; De Oliveira et al. 2000). The main risk with in vitro culture conservation over a long period lies in the induction of variations usually called somaclonal variations (Larkin and Scowcroft 1981). Indeed, in many micropropagation systems, the effect of the time spent in in vitro culture on the appearance of variant plants has often been reported (Lee and Phillips 1987; Karp 1989; Cai et al. 1990). This is the risk which we analysed over a long period through in vitro culture of oil palm somatic embryos.

As a matter of fact, the clonal propagation of elite oil palm through somatic embryogenesis leads to a morphological abnormality commonly called the "mantled" floral abnormality, which can make ramets unproductive (Corley et al. 1986). This abnormality, which is seen only once palms start flowering in the field, i.e. 2 to 3 years after planting, appear as a morphological defect of ramet flower architecture (Durand-Gasselin et al. 1993). On the flowers described as mantled, the stamen primordia develop as supplementary carpels. This abnormal morphogenesis can affect the palm to various degrees: in the more severe case all the flowers are non-functional and there is no fruit development, whilst in less severe cases only some of the female flowers are affected and they develop one or more supplementary carpels. All the research teams that have succeeded in micropropagating the oil palm have discovered such abnormalities in their respective first plantations, varying in intensity depending on the in vitro culture protocol adopted. Protocols using fast-growing calli (FGC) regenerated 100% severely "mantled" palms and most of the protocols using normally growing compact nodular calli (NGC) only resulted in a small percentage of variant palms (Corley et al. 1986; Duval et al. 1988; Paranjothy et al. 1989; Durand-Gasselin et al. 1993; Konan et al. 1995). Several causes have been suggested for the "mantled" floral abnormality in the oil palm. One hypothesis put forward is that of disrupted genome expression linked to cytokinin metabolism in the type of callus inducing somatic embryos (Besse et al. 1992). For some authors (Jones et al. 1995), the correlation between callus type and induction of the floral abnormality is not very clear, but the phenomenon would rather seem to be linked to the somatic embryo multiplication frequency and the time spent exposed to culture media containing growth regulators (Corley et al. 1986; Eeuwens et al. 2002).

The appearance of the FGC is chancy and requires numerous subcultures of the primary calli on a medium supplemented with 2,4-D. This is the reason why, at the very beginning of our project in setting up laboratories for in vitro propagation of elite oil palm, we chose the regeneration process via nodular primary calli (Pannetier et al. 1981). Moreover, our culture protocol used exogenous phytohormones for callus culture but totally avoided its use during the somatic embryo proliferation phase. This strategy was no doubt responsible for the limited abnormality rates in our plantations in which the floral abnormality has affected only 40% of the 302 clones represented and only 6% of plants seriously, over almost 1,000 ha of ramets in the field (Durand-Gasselin et al. 1993; Konan et al. 1995, 2006b; Duval et al. 1997). Results obtained on the first ramet plantations from embryo cultures maintained in vitro for less than 4 years have not shown any clear and systematic increase in the abnormality rate with the age of the embryo cultures.

To determine the maximum duration of the conservation of somatic embryos in continuous in vitro proliferation, for sustainable exploitation, without any damage for morphogenetic quality of somatic embryos or regenerated plants we launched a study in 1987 to: (1) ensure that the quality of the somatic embryo lines did not decline as subcultures were carried out over the long period of conservation and maintenance on the culture medium without phytohormones; (2) check that the long period spent in in vitro conservation by the lines did not affect the morphogenetic quality of regenerated plants; (3) prove that the age of lines conserved in vitro in the long term did not have any unfavourable effects on the stability of bunch production components (number, weight and yield) for ramets in the clonal plantations successively set up from the same source of somatic embryos.

This article covers only the aspects of the studies relating to the first two points.

Materials and methods

Plant material

The plant material used consisted of lines of somatic embryos belonging to 20 clones (in vitro regeneration from tissues of mature oil palms), the derived young plants and adult palms. The embryogenic lines belong to the in vitro collections of in vitro culture laboratory belonging to the Centre National de Recherche Agronomique (CNRA) at the La Mé Research Station in Ivory Coast. The culture of regenerated plantlets (ramets) and adult palms was carried out in the La Mé Research Station.

Somatic embryogenesis on nodular callus

Micropropagation was achieved by somatic embryogenesis on calli from leaf explants following a protocol described by Pannetier et al. (1981) and by Duval et al. (1988). After sampling the cylinder of immature leaves in the spear of the donor elite oil palm and removing the oldest leaves, leaf explants cut into small 1-cm^2 fragments were placed under aseptic conditions on a first culture medium supplemented with auxins. The basic medium was composed of MS salts (Murashige and Skoog 1962), the other compounds added to this basic medium during the various stages of the plant regeneration process are given in Table 1. For callogenesis the explants were maintained in the dark at 27°C ± 1°C. The first calli appeared at about 60 days of culture and then continued their growth on the leaf fragments (Fig. 1a). Around 100 days after the initial culture, once they were large enough, they were isolated and placed on a second culture medium (embryogenesis medium, Table 1, Fig. 1b). The concentration of free 2,4-D in that medium for embryogenesis on callus was in fact low due to the addition of charcoal (Verdeil 1993; Pan and Van Staden 1998). The calli were transferred under light (light intensity of 40 μEm^{-2} s^{-2}) with a 12/24 h photoperiod at 27°C ± 1°C.

Table 1 Culture media at various stages of the plant regeneration

Culture steps	Basic medium + major constituents of the medium required for the success of the culture stage
1. Induction of nodular compact calli (NCC) on leaf explants (callogenesis)	2,4-D (0.2 mg/l) + TCPP (0.2 mg/l) + Glucose (30 g/l)
2. Induction of embryogenic structures (embryogenesis)	2,4-D (100 mg/l) + BAP (1 mg/l) + Adenine sulphate (30 mg/l) + activated charcoal (4.5 g/l) + Glucose (20 g/l)
3. Multiplication of embryogenic structures for development into somatic embryo strains	Sucrose (30 g/l) + Casein hydrolysate (0.5 g/l)
4. Germination of somatic embryos into shoots (Germination/Caulogenesis)	Sucrose (45 g/l) + 0 g/l Casein hydrolysate
5. Rooting	Sucrose (60 g/l) + NAA (1 mg/l)
6. Weaning of rooted plants	Bed of washed or sterilised sand and kept under a plastic tunnel placed under a shade

After highly variable culture times (from 3 to 60 months), embryogenic neoformations developed on the surface of the nodular primary calli. Particular attention was paid to the quality of the calli used for embryogenesis induction, notably by eliminating systematically highly undifferentiated friable calli, called fast-growing calli (FGC), which were known to systematically regenerate plants with somaclonal variation (the "mantled" floral abnormality) (Duval et al. 1988). Under our embryogenesis conditions, these FGCs formed sporadically on the nodular calli (NC), which are well-organised compact structures (Fig. 1b) (Schwendiman et al. 1988). However, the frequency of FGC appearance on NC was very low, in <1% of NC tubes. Moreover, destructured calli or typical FGC were not used in our protocol to obtain somatic embryos.

Multiplication of somatic embryos and establishment of polyembryonic culture lines

The clumps of somatic embryos isolated from the calli (Fig. 1b) were cultured on a medium with sucrose (30 g/l) + Casein hydrolysate (0.5 g/l) without growth regulators (Table 1), in a lighted room with the same light intensity and photoperiod as that for the callus culture. The clumps of somatic embryos were extracted from the calli and placed in test tubes on the same culture medium. The clumps isolated at the same date, were grouped into populations and monitored separately based on the callus tissue origin to which they belong (Fig. 1c). In favourable cases (Schwendiman et al. 1988; Duval et al. 1988), an adventive or secondary embryogenesis phenomenon occurred, making it possible to get the multiplying embryogenic formations to evolve into proliferating lines of somatic embryos which were characterised by their permanent self-renewal potential. These lines of somatic embryos ensured the mass production of in vitro material by giving rise to exponential growth of the youngest somatic embryos, and simultaneous production of shoots through development of the oldest embryos.

Maintaining embryogenic lines in vitro over a long period plantlet production and establishment in vivo

Once established, the lines could be maintained in vitro on the same medium on which they were obtained, without the

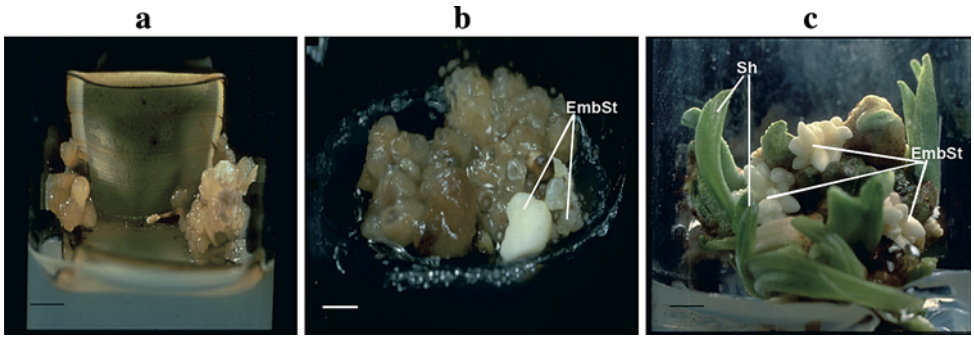

Fig. 1 Somatic embryogenesis from primary nodular calli. **a** Primary nodular calli on a young leaf explant; **b** embryogenic structures (EmbSt) appearance on nodular calli; **c** polyembryogenic lines with young shoots (Sh) on a hormone-free culture medium. *Scale bars* **a** 0.2 cm, **b** and **c** 0.15 cm

proliferation phenomenon declining. The performance of those lines in vitro culture was assessed over a period of 20 years.

The trial involved a set of 20 clones, with one line of somatic embryos per clone. Every month, the lines were subcultured on a new culture medium. Monitoring focused on the number of somatic embryo tubes created after subculturing, from the year of its isolation from the calli (year 1). The exercise consisted of maintaining a minimum stock of 48 tubes of embryos per clone in proliferation for as long as possible, but on each subculture, an attempt was made to constitute a maximum reserve of 96 tubes, until it became impossible to reconstitute that number due to the elimination of tubes of somatic embryos with an unsuitable appearance (destructured or disorganised tissues, characterised by hyperhydric tissues). Indeed, on each subculture, sorting was carried out to remove young shoots developing on the lines, contaminated tubes, and cultures with an unsuitable appearance. Monthly subculturing on a new culture medium involved only the clumps of good quality embryos, making it possible to ensure continuous embryo proliferation and shoot development.

Young shoots forming on the embryo clumps were isolated on each subculture and transferred onto a medium promoting its development (Table 1). The most developed shoots were placed on a rooting medium enriched with auxin (NAA at a concentration of 1 mg/l; Table 1). In vitro rooting was carried out in 8 to 12 weeks following a protocol previously described by Duval et al. (1988) and revised by Konan et al. (2007b). The plantlets obtained were then weaned in an acclimatisation tunnel for one month (Wuidard and Konan 1989), then transferred to a prenursery and nursery in accordance with the protocol applied to seedlings (Quencez 1982; Konan et al. 2006c).

According to our experimental protocol, around 40 plants per line of somatic embryos were produced each year in order to assess its performance under natural conditions depending on the age of the culture. However, this yearly production was not always possible for all the lines involved in the study. Laboratory constraints meant that in some years it was not possible to produce certain clones, or if they were produced they could not be planted in the field due to lack of space in the plantation.

Survival of plants after transfer to the soil and recording of the flowering data

The number of plants transferred for weaning, then to the prenursery and nursery stages, was recorded at each transfer. The ratio of the number of plants selected for transfer to the following stage to the number of plants in the previous stage amounted to the survival rate. Field observations mostly focused on the morphology of flowers spread out 2 to 3 years after planting. The quality of all the inflorescences appearing on each palm was scored by the observation method described by Durand-Gasselin et al. (1993) and by Konan et al. (1995). A clone was considered abnormal (floral variant) when at least one plant of that clone displayed flower defects. A plant was considered abnormal when at least one flower displayed abnormal morphological characteristics.

Analysis of experimental data

The plant recovery data depending on the clonal origin of the embryo line and on the age of the culture were analysed by ANOVA: age effect: 5 years, 7 years and 13 years; clone effect: 20 clones. For data on changes in the "mantled" floral phenotypic status of palms in the plantations depending on line age, trend curves were drawn for each clone in the trial, fitting a sigmoid-type function to the observed percentages of abnormal plants based on the logarithm of in vitro culture age. This can be written as:

$$\%\,\text{Abnormal plants} = \frac{100}{1 + b\, e^{-c\log(\text{age})}}$$

In basic, this formula is written as: $100/[1 + b \times \exp(-c \times \log(\text{age}))]$.

The values of parameters b and c, corresponding to the adjustment of the least squares, were calculated by the SAS software NLIN procedure (SAS Institute Inc. 2006).

Results and discussion

Influence of time spent in vitro on changes in the morphological appearance of proliferating polyembryogenic lines

On monthly subculture, the number of test tubes containing somatic embryo lines in a good state of proliferation (Fig. 2a) was counted over the conservation period, which lasted up to 20 years. However, for the sake of simplification, only the stock of tubes existing for the January count (chosen for convenience) of each year is presented per clone (Table 2).

Table 2 shows that maintaining somatic embryo lines in continuous in vitro proliferation over a long period led to a gradual reduction in the tissue quality of the proliferating embryo lines from the ninth year onwards (Fig. 2b, c). Unfortunately, it was not possible to assess the organogenesis ability of the lines, in terms of germinated plant yields, in the trial over the conservation period. However, observations carried out during subcultures showed that the test tubes with lines displaying degraded tissue did not usually contain any normal shoots (Fig. 2b, c). However, the deterioration in line quality did not involve all the clones. Of the 20 clones tested, only 6 (30%) (LMC 044, 051, 074, 106, 165 and 172) maintained their maximum useful number of 48 tubes of somatic embryos over the entire observation period (20 years) (though production circumstances may have made it possible to increase that number), indicating that the proliferating lines had maintained their multiplication and plant regeneration potential intact, like the lines with good tissue quality (Fig. 2a). For the other 14 clones, the number of somatic embryo tubes required remained intact up to the end of the 8th year, a period after which that number could no longer be reconstituted after each subculturing operation, indicating that the lines had started to lose their multiplication potential. The deterioration had begun for 15% of the clones (3 clones: LMC 052, 144 and 167) by the ninth year of proliferation, then gradually affected the other clones each year: 4/20, 10/20, 12/20 and 14/20 in years 11, 13, 15 and 16, respectively.

The reasons for the variable performance of lines kept in in vitro culture were not identified in this study. However, the clone effect (parental origin of the leaf samples) has always been shown to be statistically significant in the performance of the oil palm in vitro regeneration process (Duval et al. 1988; Konan et al. 2007b).

In plant micropropagation, many authors attribute this clone effect or genotype effect to differences in the endogenous level of growth regulators or to the reaction capacity, which differs from one individual to the next (Schween and Schwenkel 2003; Winkelmann and Serek 2005).

Apart from contaminated cultures and young developing shoots, which were naturally discarded during subcultures, most of the cultures eliminated involved destructured haustorium type tissues characterised by a hypertrophy of the part corresponding to the embryo haustorium (the expanding distal portion of the cotyledon) exhibiting an apparent chlorophyll deficiency. The cultures shown in Fig. 2b and c were systematically eliminated. Those tissues were assimilable to typical hyperhydric malformations of vitrified tissues described in in vitro culture (Gaspar 1995; Kevers et al. 1984), with vitrification being considered as the morphological response to a non-traumatic stress (excess water, cytokinins, certain ions, etc.), or also as the consequence of the disorder of certain metabolic pathways

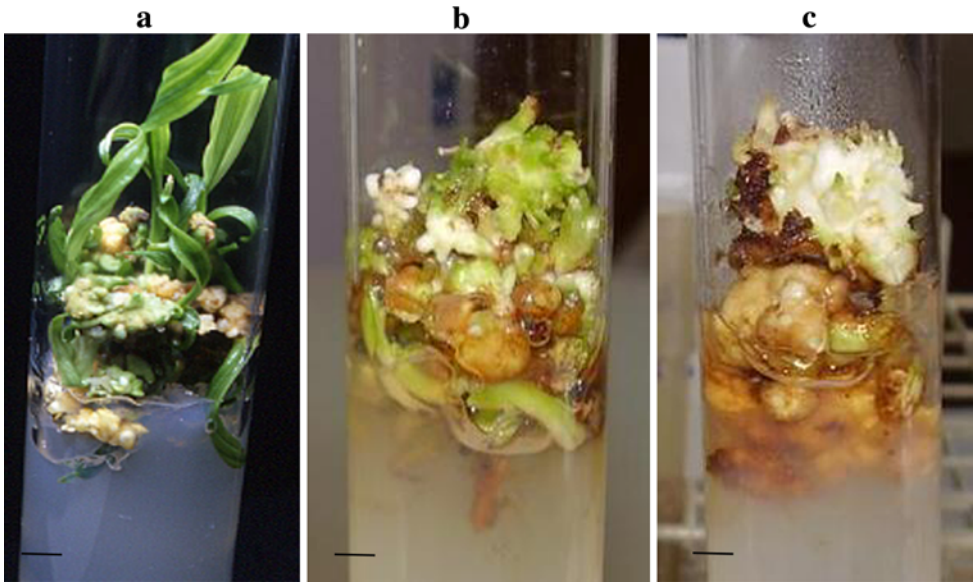

Fig. 2 Typical cases of polyembryonic cultures at the end of the proliferation cycle. **a** Strain with very good quality, endless proliferation, with great germination potential; **b** proliferating strain with some clumps of somatic embryos in good multiplication condition, and developing shoots, but containing some deteriorating tissue structures which will be eliminated during subculturing; **c** destructured strain, with degraded tissue quality, which will be totally eliminated from the culture collection. *Scale bars* **a** 0.3 cm, **b** and **c** 0.25 cm

Table 2 Change, for each clone, in the number of tubes containing strains of somatic embryos with good tissue quality, kept in multiplication after the January subculture of each year, from year 1 of the isolation of the original embryogenic formations

Clones	Time taken to create the somatic embryo strain (Years)	Age of the somatic embryos maintained in in vitro multiplication since year 1 of isolation from the original callus																			
		Number of tubes of embryos created when embryos were isolated from the calli																			
		Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10	Year 11	Year 12	Year 13	Year 14	Year 15	Year 16	Year 17	Year 18	Year 19	Year 20
LMC 009	4	7	11	20	24	32	38	48	48	48	48	42	25	*13*	*9*	*3*	*0*	*0*	*0*	*0*	*0*
LMC 022	4	2	18	21	48	48	80	66	96	96	73	96	60	26	*12*	*10*	*7*	*0*	*0*	*0*	*0*
LMC 026	3	4	12	41	48	48	48	48	57	48	48	24	96	96	96	33	*16*	*4*	*0*	*0*	*0*
LMC 044	3	4	16	37	37	48	96	96	53	45	29	65	64	49	57	62	78	96	96	96	96
LMC 051	2	11	33	45	96	48	48	96	96	96	96	96	96	96	96	96	96	96	96	96	96
LMC 052	3	6	19	48	48	48	46	48	34	*16*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 063	4	2	17	29	41	48	55	96	96	96	96	78	49	19	24	*14*	*6*	*0*	*0*	*0*	*0*
LMC 074	4	4	9	25	48	33	48	96	96	56	96	96	53	96	96	96	96	96	96	96	96
LMC 106	2	4	22	26	24	48	48	48	48	48	48	48	96	96	96	96	96	96	96	96	96
LMC 107	4	1	13	16	19	26	48	48	48	47	*19*	*8*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 130	3	2	12	27	33	27	37	48	36	44	32	*8*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 144	1	6	36	41	48	48	42	30	23	*10*	*4*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 152	2	1	14	21	35	30	48	28	76	89	56	36	21	*7*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 158	2	6	18	25	35	35	37	81	56	32	*19*	*11*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 159	2	2	48	42	48	48	48	23	96	71	43	49	31	*17*	*9*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 161	3	2	16	24	24	28	48	48	34	35	22	33	27	45	42	21	*9*	*3*	*0*	*0*	*0*
LMC 165	2	1	32	46	48	48	48	35	48	41	63	96	96	96	96	96	96	96	96	96	96
LMC 167	2	8	23	38	48	96	49	48	35	*17*	*9*	*3*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
LMC 172	2	5	31	34	40	48	48	48	46	52	58	76	83	96	96	96	96	96	96	96	96
LMC 174	2	3	47	48	48	48	47	48	48	36	24	25	*7*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Number of clones represented by less than 20 tubes of somatic embryos			0	0	0	0	0	0	0	3	4	6	7	10	11	12	14	14	14	14	14
%			0	0	0	0	0	0	0	15	20	30	35	50	55	60	70	70	70	70	70

Although subculturing of the embryo strains on new medium was carried out each month for the 20 years that in vitro conservation lasted, the data indicated in the table represent the number of tubes of embryos kept after the January subculture. The italicised data indicate the number of tubes still being kept in vitro after sorting tubes containing strains in a state of degradation

(sugar, nitrogen and ethylene). Under our in vitro culture conditions, the lines with a vitrified appearance increased its mass, but this growth did not correspond to an increase in the number of embryonic structures. Consequently, these cultures were incapable of developing into shoots. The gradual decline in the quality of those lines depending on the time spent in proliferation, suggests a problem of tissue degenerescence, which is known in cell culture as a senescence phenomenon (Okamura et al. 1975; Codron et al. 1979; Muhitch and Fletcher 1985; Gan and Amasino 1995, 1997; Carimi et al. 2004). Several authors have blamed growth hormones, and more particularly polyamines (putrescine and spermidine) in the expression of such disorganised cell proliferation (Slocum et al. 1984; Faure et al. 1991). Although our somatic embryo multiplication medium did not contain these compounds, it is, however, possible that the behavioural disorder of the somatic embryos could be attributed to the effect of organic nitrogen incorporated into the proliferation medium in the form of casein hydrolysate. We have observed that the use of higher amounts of casein hydrolysate led to an increase in the mass of embryo lines, but cultivated on media containing this compound at concentrations of 1, 1.5 or 2 g/l, the embryo clumps became destructured like those on Fig. 1c and did not give any shoots after few subcultures (data not shown).

The degraded state of the somatic embryo lines seemed to correspond to that described on somatic embryos of Vitis *vinifera* that had reached the development stage called T+ (Faure 1990; Faure et al. 1991). At that stage of development, somatic embryos are characterised by anarchic cell development typical of tumour cells in which the polyamine level is very high (Bagni and Serafini-Fracassini 1979). Polyamine production is also typical of vitrified tissues (Hagège et al. 1990; Le dily et al. 1993). It is highly possible that on a culture medium rich in organic nitrogen, the oil palm somatic embryo lines accumulate excessive reduced nitrogen (NH_4^+ ion) over the years, which eventually causes stress inducing the synthesis of polyamines, the metabolism of which would seem to be involved in the behavioural disorder of the lines. Such a phenomenon has been demonstrated in sugar beet, a plant for which obtaining vitrified calli with a hyperhydric appearance has been associated with nitrogen metabolism disruption (Le Dily et al. 1993). Other compounds, such as phenols, can also accumulate with the age of the lines, as reported by certain authors (Muhitch and Fletcher 1985), but probably in our case the excessive nitrogen that lies behind polyamine synthesis seemed to be one of the most likely causes of the gradual decline in culture quality as the time maintained in vitro increased.

The mechanism bringing into play polyamine synthesis in plants has been studied from different viewpoints and some authors have reported a direct interaction with ethylene (Lee and Chu 1992; Tamai et al. 1999), with the biosynthesis of polyamines and of ethylene taking place via a common precursor *S*-adenosyl methionine (SAM). Ethylene is also produced by tissues and organs in response to environmental stress, such as wounds, exposure to cold and heat (Konze and Kwiatkowski 1981; Kimmerer and Kozlowski 1982; Tong and Yang 1987).

Corbineau et al. (1990) have shown that clumps of oil palm somatic embryos are able to produce ethylene at 27°C and that ethylene emission can be a good stress indicator. In fact, it is at 27°C that the somatic embryo lines were maintained under our in vitro culture conditions. It is, therefore, possible that frequent separation of the lines with a scalpel and its cleaning for transfer to fresh medium, which has resulted in repetitive wounding, led to substantial ethylene emissions. In the most susceptible clones, regular accumulation of ethylene over time must have triggered the production of polyamines responsible for degradation of the conserved tissues.

Effect of line age on the survival of plants transferred to natural conditions

The results (Table 3) indicate that, overall, the plant survival rate after transfer fell steadily in line with the age of the laboratory cultures. Nevertheless, recovery rates of more than 50% were observed until 7 years of line conservation but beyond that date, the drop was significant each year, falling from almost 40% at 9 years and reaching <30% at 13 years (effect of line age on recovery: $F(4,71) = 12.535$; $^{***}P < 0.000$). The 50% plant recovery observed in this experiment for lines kept in vitro for up to 7 years confirmed our earlier results although those results concerned the first years in which ramets were planted. Likewise, the variability in clone performance for ex vitro recovery observed here (Table 3) on the plants further confirmed our earlier results (clone effect: $F(19,72) = 2.298$; $^{**}P = 0.009$). Most of the plants eliminated from old lines conserved in vitro for more than 5 years exhibited developmental defects in the prenursery (Fig. 3) and nursery (Fig. 4), rarely seen on the ramets derived from the first outputs from the same clones: spindly plants with etiolated leaves, plants with jagged and fused leaves, called "self-pruning plants" (Jacquemard 1992). These results seem to indicate that deterioration in the quality of lines generating shoots could have taken place at a precise moment of the conservation period, which demonstrates that the age of oil palm lines has a negative effect on the recovery capacity of plants undergoing acclimatisation. The plant loss rate reached 60% in the ninth year of conservation; that rate then evolved, reaching 63% then 76% after 11 and 13 years, respectively. This observation

Table 3 Percentage recovery of regenerated plants under natural conditions depending on the time spent by the somatic embryo strains in vitro

Clones	Recovery at 5 years			Recovery at 7 years			Recovery at 9 years			Recovery at 11 years			Recovery at 13 years		
LMC	Number T0[a]	Number end	% Recovery	Number T0	Number end	% Recovery	Number T0	Number end	% Recovery	Number T0	Number end	% Recovery	Number T0	Number end	% Recovery
009			nd	178	53	30	230	46	20	148	25	17	651	33	5
022			nd	150	99	66	246	170	69	170	111	65	194	87	45
026			nd	278	111	40	132	54	41	78	31	40	161	56	35
044	300	159	53	298	164	55	260	88	34	241	77	32	34	11	33
051	270	154	57	274	170	62	240	74	31	590	177	30	553	182	33
052	82	52	64	204	133	65	159	86	54	29	17	57	99	40	40
063	152	94	62	191	101	53	97	31	32			nd			nd
074	215	127	59	122	70	57	113	53	47	95	36	38	204	49	24
106	100	57	57	18	9	52	118	35	30			nd			nd
107	21	19	90	499	324	65	80	51	64			nd			nd
130	117	80	68	116	80	69	138	72	52			nd			nd
144	13	7	53	79	39	49	18	4	22			nd			nd
152	122	76	62	270	143	53	36	6	16			nd			nd
158	113	68	60	117	63	54	101	47	47			nd			nd
159	132	57	43	140	74	53	161	87	54	82	46	56			nd
161	155	113	73	68	41	61	58	19	32	17	3	18			nd
165	190	146	77	104	60	58	85	55	65	57	39	68			nd
167	335	198	59	168	84	50	372	119	32			nd			nd
172	138	97	70	53	34	64	59	10	17			nd			nd
174			nd	29	21	71			nd	21	7	33			nd
Total	2,455	1,503	61%	3,356	1,873	56%	2,703	1,107	41%	1,528	569	37%	1,896	458	24%

The recovery percentages were calculated per clone and per duration of in vitro strain conservation, each sample of ex vitro plants was considered as a replicate. The ANOVA indicated the following results: effect of strain age: $F(4,71) = 12.535$; $^{***}P < 0.000$; clone effect: $F(19,72) = 2.298$; $^{**}P = 0.009$

[a] Number of plants transferred under natural conditions; *nd* recovery % not determined

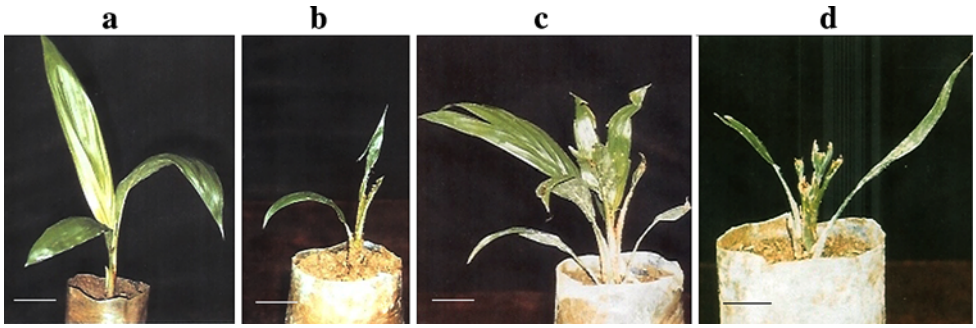

Fig. 3 Oil palm ramets in the prenursery. **a** Normal plantlet; **b** plantlet with jagged leaves and slightly fused lamina; **c** "self-pruning" plantlet with jagged leaves stuck together 1st stage; **d** "self-pruning" plantlet, final stage. *Scale bars* **a** 7 cm, **b** 5.4 cm, **c** 4.5 cm, **d** 3.5 cm

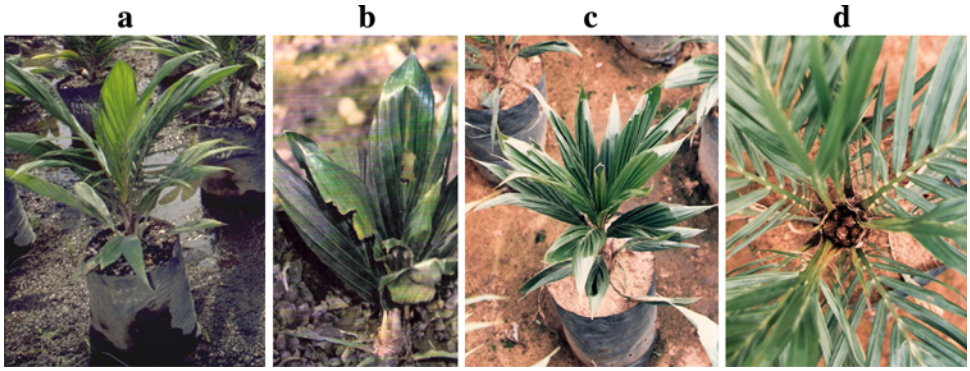

Fig. 4 Oil palm ramets in the nursery. **a** Normal plant; **b** "self-pruning" nursery plant with jagged leaves; **c** plant with rigid and upright leaves; **d** plant the vegetative apex of which has turned into a terminal inflorescence

seems to indicate that line age affected the physiological quality of regenerated plants, confirming the results obtained by Rival et al. (1997a), who reported that, in oil palm, the physiological quality of ramets undergoing acclimatisation is the most decisive criterion for recovery after planting out, though some losses were also linked to problems with the quality of roots formed in vitro (Wuidard and Konan 1989).

Variation in "mantled" abnormal palm rates depending on line age

The observation results were interpreted separately for each clone, based on the curves showing trends for the abnormality rates (Fig. 5). The variation in palms found to be abnormal in the successive plantations established from the same origin of embryo lines depended on the clone, making it possible to group all of them into two major categories, depending on its tendency, or not, to regenerate abnormal plants: (1) clones displaying a stable tendency which gave normal plants in all the plantations successively installed, even after maintaining the laboratory cultures in vitro for 20 years (12/20 clones: 60%); (2) clones for which the abnormality was induced early and was visible in the first plantations setup (4/20 clones: 20%; LMC 051, 052, 130 and 152); along with those for which the "mantled" floral abnormality was seen late, only involving plants derived from old lines after more than 6 years of in vitro conservation (4/20 clones: 20%; LMC 063, 106, 107 and 144).

The distribution of the proportions found in this experiment, between normal (60%) and abnormal (40%) clones confirmed the previous conformity frequencies for our clones in plantations (Durand-Gasselin et al. 1993; Konan et al. 1995; Duval et al. 1997). When considering the affected clones more specifically, the increase in "mantled" palm rates in successive plantations in line with the time spent by the embryogenic lines in vitro also tallied with the results obtained by Corley et al. (1986), though its results were obtained by maintaining embryos on media containing auxins and cytokinins, although in our case two types of abnormal clone behaviour were revealed: clones the embryoid lines of which regenerated "mantled" abnormal plants right from the first plantations, and clones that were only affected later, i.e. after the material had been kept in vitro for 6 to 10 years. Several causes have been suggested for the "mantled" floral abnormality in the oil palm (Rao and Donough 1990; Rival et al. 1997b; Matthes et al. 2001). The hypothesis of an alteration in the level of genomic DNA methylation linked to the metabolism of phytohormones during the in vitro regeneration process has been accepted (Jaligot et al. 2000, 2002; Eeuwens et al. 2002). The hormones involved, along with the culture stages concerned have been studied from different angles. It has been shown that the disruption could be induced at the callus stage by the level of 2,4-dichlorophenoxyacetic acid (2,4-D)

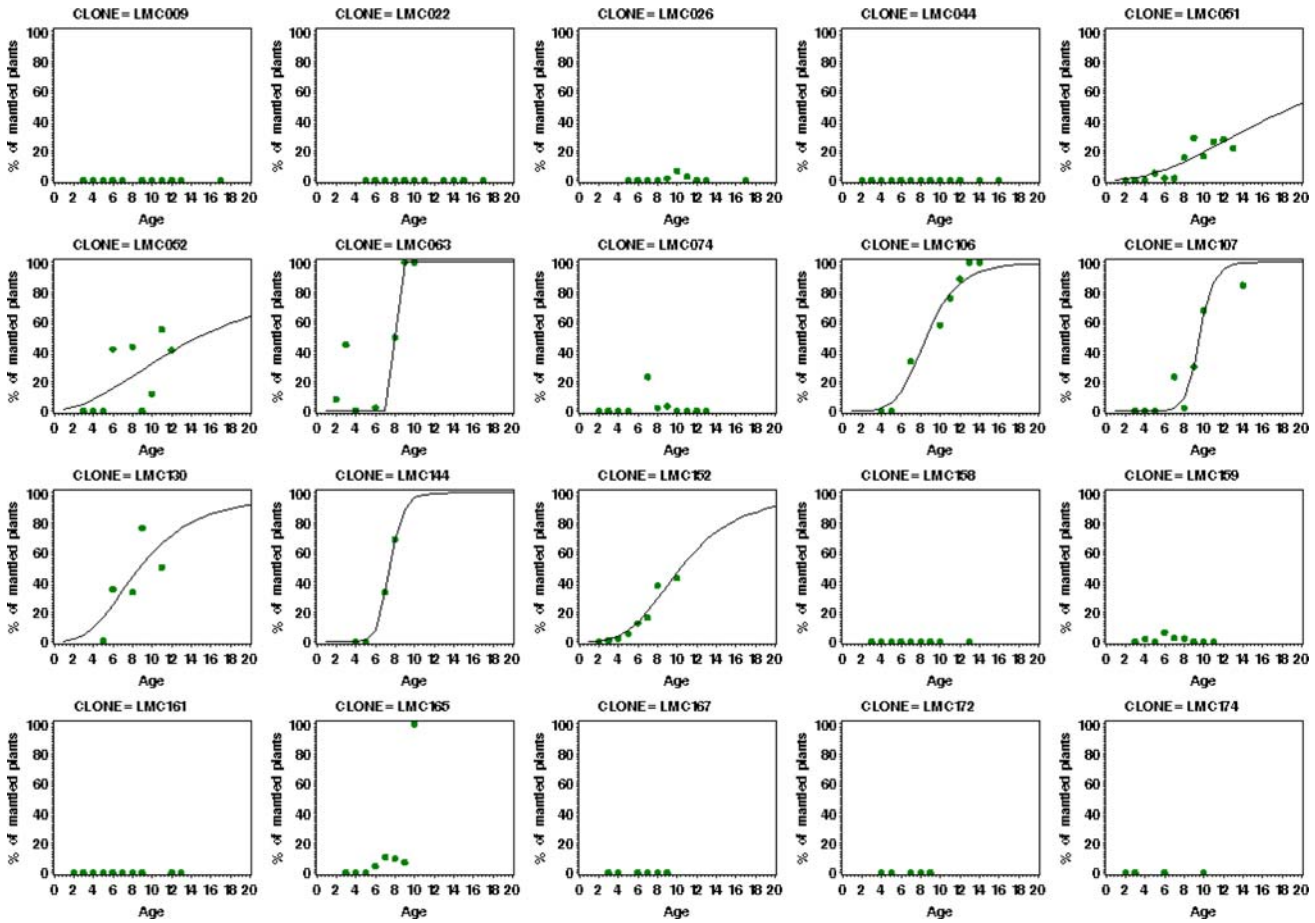

Fig. 5 Percentage of "mantled" plants in the field depending on the age of the strains conserved in vitro and overall trend for floral abnormality per clone

(Soh 1987). In carrot, that synthetic auxin has been held responsible for changes in genomic DNA methylation status (LoSchiavo et al. 1989). In addition, there have been reports of that auxin having an influence on the disorganisation of nodular calli (NC) into fast-growing friable calli (FGC) in the oil palm (Duval et al. 1988). Use of nodular calli leads to an abnormal palm frequency of 5 to 10%, whereas embryogenesis achieved with fast-growing friable calli (FGC) leads to the regeneration of plants that are systematically 100% abnormal. The hormone content between the two types of calli also showed that the auxin:cytokinin ratio (IAA/Zeatine glucoside and riboside) was much higher in FGC than in NC (Besse et al. 1992). Consequently, the endogenous auxin/cytokinin ratio in the type of embryogenic calli used could be a marker of the "mantled" abnormality (Jones et al. 1995; Besse et al. 1992).

In our culture conditions, FGC were systematically eliminated as soon as they were seen. That no doubt contributed to reducing the relatively low proportion of abnormal plants in our culture system but some FGC existed that were not visible to the naked eye and which may have led to somatic embryos regenerating abnormal plants from the first year after planting out. In all cases, observation of the abnormality in the first year after a clone was planted may suggest that the somatic embryo had already been affected at its induction on the callus. It is, therefore, normal that the multiplication of these variant embryos, the progeny of which would necessarily have inherited the variant trait, led to an increased frequency of abnormal palms in the subsequent plantations. Similar observations showing the existence of a positive correlation between the number of subcultures (in vitro duration) and the percentages of abnormal plants have been reported for banana (Larkin and Scowcroft 1981; Côte et al. 1994). However, the appearance of the "mantled" floral variation several years after, in plantations successively setup using the same line of somatic embryos maintained all the time in vitro on a culture medium without any phytohormones cannot be explained, if it is accepted that this type of medium entails fewer risks than media containing phytohormones (NAA, BAP and Kinetin) used by Eeuwens et al. (2002). Nevertheless, the habituation of the polyembryogenic lines which can be due to a high synthesis of endogenous auxin may induce abnormal floral morphogenesis in the regenerated

plants. Unless the induction effect of that in vitro variation was masked or delayed for several years running, it is tempting to suggest that factors other than endogenous growth regulators might lie behind the genetic and/or epigenetic disruptions seen in clones affected at a later stage.

Probably, the variation triggered at a later stage may have been brought about by other factors linked to the long somatic embryo conservation period, and to the very maintenance procedure itself, which calls for regular monthly subcultures. The direct implication of ethylene produced by proliferating tissues (in response to wounding when cleaning the clumps of somatic embryos with a scalpel), in endogenous biochemical reactions, can be put forward as a probable explanation for the induction of floral variation, as this hormone is also considered to promote flowering (Eeuwens et al. 2002; Dukovski et al. 2006). Likewise, the metabolism of the polyamines accumulated in line with the age of the lines, under the effect of ethylene, can also be suggested as a possible source of epigenetic disruptions causing the "mantled" floral abnormality. Indeed, in plant cells, polyamines would seem to be involved in major cell processes, such as mitosis, protein synthesis, DNA replication (Kakkar and Sawhney 2002). Under physiological pH conditions, it has been reported that polyamines behaved like cations and would seem to interact with anionic macromolecules, such as DNA and RNA, phospholipids and certain proteins (Kumar et al. 1997). Some enzymes (RNAse, ATPase and protease) inhibiting or stimulating properties have also been acknowledged in polyamines (Atman 1982; Reggiana et al. 1992). Might those polyamines act on molecular functions, leading to poor transcription of certain genes linked to the "mantled" abnormality in the oil palm? The mechanism of their action on the genome, along with the type of alteration caused, need to be clarified. That would provide some further information on the type of genetic expression linked to the "mantled" floral abnormality.

Conclusion

The effect of the multiplication duration on a free phytohormone medium, but rich in nutrients (casein hydrolysate and sucrose), on the behaviour of oil palm somatic embryos maintained in proliferation in vitro for almost 20 years was assessed. Over time, with numerous subcultures, culture conditions in a global sense proved to be depressive both for the morphogenetic quality of the lines and for the plant survival rate after planting out. The age of lines maintained over a long period also displayed an effect conducive to induction of the "mantled" floral abnormality, but no clear relation was found between the lines that proliferated for a long time whilst maintaining cultures in vitro with good tissue quality, lines with a good recovery rate after planting out and those giving rise to true to type palms in the field. The survival of oil palm ramets under natural rearing conditions has been attributed to problems with the general development quality of the plant at the time of transfer (Wuidart and Konan 1989; Rival et al. 1997a, b), whereas the "mantled" floral abnormality was linked to molecular regulation problems in genome expression relating to hormone metabolism (Besse et al. 1992; Jones et al. 1995; Jaligot et al. 2002; Rival and Parveez 2004).

To our knowledge, little work has been published on such a long period of in vitro conservation of embryogenic material, quantifying the variations that might occur either in proliferating cultures maintained in vitro or in plants transferred to natural conditions in the field. Although many studies have been carried out on the determinism of the "mantled" somaclonal variation in the oil palm, the hypotheses we put forward regarding the various factors acting both on the quality of proliferating tissues and on the floral conformity of clonal regenerants, reveal new avenues of research on early markers of this abnormality, which still remains a handicap for the commercial dissemination of clonal material. The susceptibility of clones to ethylene emission will have to be one line of study in the search for a physiological marker linked to the somaclonal variations observed. If it were to be identified, the marker could serve as a tool in controlling the conditions responsible for culture degradation and induction of the floral morphological abnormality.

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