Scaling-up micropropagation of palms: the example of oil palm

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1. Introduction

Clonal propagation of oil palm (*Elaeis guineensis* Jacq.) through somatic embryogenesis has been achieved by several research groups (Corley *et al.*, 1977; Pannetier *et al.*, 1981; Paranjothy, 1984), and its merits have been well discussed from a theoretical angle (Soh, 1986). The French ORSTOM-CIRAD group has developed a micropropagation process up to the pilot scale in four production units spread over three producing countries. *In vitro* vegetative propagation has led to the production of more than one million clonal plantlets to date. The technique has been proven by field performances on more than 2,500 ha of clonal plantations. Scaling-up of the current process has revealed several bottlenecks that are now limiting the commercial development of the tissue culture process. The main difficulties encountered at the pilot scale are: (i) the production costs and (ii) the occurrence of variant palms (*ca.* 5%) in embryogenically derived plant material. The production costs are still high because the process remains very labour- intensive and proliferation rates are overall rather slow. The development of oil palm embryogenic cell suspension has been achieved and will certainly enable the large-scale propagation of selected genotypes at low cost. Studies have been conducted in order to improve medium-term conservation of somatic embryos derived from embryogenic suspensions and the vigour of the regenerated plant material. This new process is currently assessed in terms of genetic fidelity.

In order to understand the phenomena underlying the determinism of somaclonal variation, molecular approaches are now being developed in our group, involving studies on DNA structure together with patterns of genome expression in the regenerated plant material. The limitations in scaling-up that have been identified

at the pilot scale have stimulated the implementation of new research programmes in the fields of plant and seed physiology and molecular biology. Results expected from these programs will be used for the setting up of an improved production protocol, based on the culture of embryogenic suspensions and certified with a quality control for genetic fidelity.

The aim of the present paper is to present the different steps that have been cleared by the ORSTOM-CIRAD French group and its partners in producing countries during the implementation of scale-up procedures for the micropropagation of oil palm through somatic embryogenesis.

2. Regeneration protocol and technology transfer

Cloning of oil palm is performed by somatic embryogenesis on calli of leaf origin, using a protocol already described (Pannetier *et al.*, 1981; Duval *et al.*, 1995b). Callogenesis is obtained in the dark (27°C) on an initial culture medium containing 2,4-D. After 12 to 20 weeks, the calli are collected and placed on a second culture medium with a lower 2,4-D concentration than the previous one. After a variable period, embryogenic formations develop on the surface of the calli. They are isolated and cultured on a growth regulator-free medium. At best, an adventive embryogenesis mechanism is triggered, enabling the formation of embryogenic neoformations. This leads to indefinite culture proliferation. Alongside this phenomenon, the oldest somatic embryos develop into shoots, which are collected and placed on an NAA-enriched medium for eight weeks to allow for root development. Then they are transferred to the acclimatisation stage, and subsequently to the pre-nursery, according to the protocol applied to seedlings.

The transfer of technology from the research laboratory to the pilot production unit scale was initiated in 1982. A production laboratory designed for the annual production of 250,000 clonal plantlets was set up in Côte d'Ivoire (West Africa). In 1985-1986, two laboratories based on the same model were opened in Indonesia, with two different partners, one from the public sector (IOPRI - Indonesian Oil Palm Research Institute) and one from the private sector (SOCFINDO - Socfin Indonesia). At the same time, one laboratory was established in Malaysia, in collaboration with a national development agency (FELDA - Federal Land Development Authority). A subsidiary of CIRAD for the commercial development of the process (TROPICLONE SA) was created in 1987 and a production laboratory was opened in France.

3. Assessment of the pilot process

Oil palm clonal *in vitro* propagation has been practised since 1981 in Côte d'Ivoire in the IDEFOR-DPO (Institut des Forêts - Département des Plantes Oléagineuses) Laboratory, in collaboration with ORSTOM and CIRAD-CP. The regeneration process has been assessed on a large scale with steady planting of clonal material. After more than 15 years of activity, the IDEFOR-DPO Laboratory has obtained a considerable amount of data on the performance of the process (Duval *et al.*, 1995b; Duval *et al.*, 1997).

Since 1981, 460 palms have been sampled for tissue culture, including palms of *E. guinensis* var. pisifera, backcrosses and *in vitro* regenerants. All the palms sampled in La Mé using our standard procedure have produced calli on foliar explants. Success rates in callogenesis may vary according to the genetic origin of the mother palm. Palms from *Deli x La Mé* origin produced calli on 31% of explants on average, whereas this percentage reached only 7% and 9% respectively in palms of *Deli x Nifor* and *Deli x Yangambi* origin. Within a given cross, callogenesis rates are highly variable ranging from 20% to 175% depending on the genetic origin. Embryogenesis on nodular callus was obtained with an overall success rate of 87%. This phenomenon occurs at a very slow rate (taking up to 2 years) and remains difficult to control. Success rates during the late phases of the micropropagation process were as follows: 83% during acclimatisation, 79% during pre-nursery and 78% during nursery. Thus the overall success rates were 62% from pre-nursery to nursery and 51% from hardening to nursery (end). To date, the total production of the La Mé Laboratory since its opening in 1981 has reached 750,000 plantlets, originating from 216 different clones. The overall area planted with clonal material (genetic trials + commercial plots) in Côte d'Ivoire has now reached 800 ha (thus *ca.* 110,000 palms).

In the clone trials carried out in Côte d'Ivoire (Duval *et al.*, 1997), 16 out of 22 clones produced substantially more than the seed control (L2TxD10D), with yield increases ranging from 10% to 54%. Furthermore, 15 clones produced at SOCFINDO, IOPRI and ORSTOM were superior to the control, with yield increases of between 8% and 44%. The identification of several clones with high yield potential confirms the forecasts made by breeders (Meunier *et al.*, 1988) and the merits of vegetative propagation of the best palms obtained in progeny tests. However, our results from scaling up of *in vitro* protocols and field trials have shown the different obstacles to the dissemination of the technique.

4. Limitations for mass production

The scaling-up of the tissue culture process at various locations with different partners has revealed almost everywhere the same difficulties. Problems have generally occurred in two major fields.

4.1. Production costs

Tissue culture of palms is generally time-consuming and the biological events involved in each step of the process progress very slowly, as it has already been reported for coconut (Verdeil and Buffard-Morel, 1995). The total time necessary between the sampling of the mother palm and the hardening of the first batch of plantlets is 18 months on average (Duval *et al.*, 1995b). The latter has a negative impact on the production cost, as overheads for the propagation facilities remain very high. Because of the very high production costs, the selling price of clonal plantlets could not be lowered to less than 5 times the price of selected seeds (2 to 3 USD). The process as a whole is very labour intensive, as several steps require very skilful manual operations (selection of calli and competent embryogenic structures, separation of shootlets before *in vitro* rooting, etc.). Furthermore, the regeneration protocols currently being developed are unsuitable for large-scale production, *i.e.* 104-105 units per clone per year. At present, only a few clones can reach an annual production of around 104 plants. Furthermore, it appears that for a given clone, the culture and production management approaches used determine the availability of a given clone and when it can be supplied. Thus, customer requirements have to fit in with the vagaries of production, which is not acceptable from a commercial point of view.

4.2. Genetic fidelity of regenerants

Evaluation in the field has revealed the occurrence of a small percentage (*ca.* 5%) of variant palms that show an abnormal flower development (Duval *et al.*, 1995b). This character, originally referred to as 'mantled' by Corley *et al.* (1986) has been characterised as a feminisation of the male parts in flowers of both sexes. The alteration results in an abnormal floral development, by modifying two of the inner whorls. This leads to abnormal morphogenesis of the fruit, which bears supernumerary carpels around the drupe; hence the term 'mantled'. The severity of the phenomenon varies and may have only a slight influence on oil yields if it does not prevent fruit set (as it is the case with 'slightly mantled' variants), or leads to abortion of flowers, thus to complete sterility of the palm ('severely mantled'). The early detection of 'mantled-type' somaclonal variants is thus today of critical concern in oil palm clonal micropropagation.

5. Research programmes related to scaling-up procedure

5.1. Embryogenic suspensions/artificial seeds

Progress has recently been made in plant production through somatic embryogenesis, particularly by developing systems based on the artificial seed concept (Redenbaugh, 1993), in which the somatic embryogenesis process is used to produce individual embryos with relatively synchronous development. Embryo development can be halted at a given stage, either by following the natural procedure that occurs during zygotic embryo quiescence or by using artificial methods, such as low temperature storage. The somatic embryos, which are often encapsulated with antifungal/nutritive coating are then used as seeds, either *in vitro* or sown directly in the field (Fuji *et al.*, 1992). Embryogenic oil palm cell suspensions have been initiated by several authors (Teixeira *et al.*, 1990; Touchet *et al.*, 1990).

Research work has been initiated in our group (Touchet *et al.*, 1990, 1991; Duval *et al.*, 1995a, b) in order to develop new methods of automation and scaling-up for oil palm micropropagation (Bajaj, 1991). To date, embryogenic suspensions have been successfully isolated for more than 20 clones. The average concentration was *ca.* 105 cell clusters per litre with a multiplication factor reaching 4x per month. These characteristics allow mass propagation. Sondahl (1991) reported on the successful culture of oil palm cell suspensions in bioreactors. Field trials are under way for the assessment of clonal fidelity in plantlets originating from cell suspension cultures.

Somatic embryo-derived plantlets are generally less vigorous than plantlets resulting from zygotic embryo germination. This relative weakness may be the consequence of an incomplete maturation of somatic embryos (Crouch, 1982). Storage proteins might be appropriate markers for the assessment of the maturation of somatic embryos (Redenbaugh *et al.*, 1986) and thus their ability to withstand desiccation. In oil palm, our research work aims to understand the patterns of storage protein accumulation in somatic embryos. This understanding will aid to: i) improve the vigour of the *in vitro* regenerated plants produced through embryogenic suspensions and ii) carry out medium term conservation of embryos at room temperature as encapsulated artificial seeds (McKersie *et al.*, 1995) or alternatively by using cryopreservation (Engelmann, 1991; Dumet *et al.*, 1993). Recent work in our group (Morcillo *et al.*, 1997b) has shown that 7S globulins, which are the major storage proteins in oil palm zygotic embryos, might potentially serve as markers in the study of somatic embryogenesis.

Oligosaccharides are generally involved in the tolerance of desiccation in embryos (Le Prince *et al.*, 1993). These compounds have been demonstrated to play a central role in the creation of a vitrous state and in the protection of the cellular structure (raffinose and stachyose) against crystallisation of solutes. The [sucrose/(raffinose + stachyose)] ratio thus may be considered as a reliable indicator of the capacity of embryos to withstand desiccation.

In oil palm zygotic embryos, this 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.*, 1995; Chabrillange *et al.*, 1996). In oil palm somatic embryos, resistance to desiccation is improved when embryos are treated with ABA and sucrose at the end of the maturation phase (F. Aberlenc-Bertossi, pers. comm.).

5.2. Physiology of vitroplants

Several studies have been conducted in order reduce acclimatisation losses, which have an important impact on production costs, because they occur at the end of the tissue culture process. The *in vitro* photosynthetic parameters of the embryogenically-derived plant material have been measured throughout the process, with the aim of characterising the physiological status of the *in vitro* regenerated plants and thus optimising success rates during acclimatisation (Rival *et al.*, 1994; 1996; 1997b; 1997c). The latter work showed that active photosynthesis could be measured very early during the micropropagation process. Losses during acclimatisation to natural growing conditions were thus thought to be mainly caused by poor *in vitro* rooting in various cases. Studies on changes in peroxidase activity during *in vitro* rooting of oil palm clonal plantlets have led to the implementation of an efficient rooting protocol (Rival *et al.*, 1997e). This improved procedure has been successfully developed in production units.

5.3. Genetic fidelity

5.3.1. Biochemical markers

Several potential biochemical markers of the 'mantled' abnormality in oil palm have been investigated by our group. Polypeptide patterns (Marmey *et al.*, 1991) and endogenous cytokinins (Maldinet *et al.*, 1986; Besse *et al.*, 1992) have been studied. Nevertheless, it has been very difficult to assess the validity of such markers on a large number of samples, because of the lack of repeatability (in the case of proteins) or the high cost (in the case of endogenous cytokinins) of such estimations.

5.3.2. Ploidy level

Flow cytometric analysis has revealed that embryogenic calli and plants showed the same ploidy level (Rival *et al.*, 1997a). No variation in the ploidy level between seed-derived palms and 3 different types of calli originating from foliar explants, namely nodular compact calli, fast-growing calli and friable calli, was observed. Since fast-growing calli (FGC), already identified as a source of 'mantled' phenotype variants, did not show any difference in their ploidy level, these results are consistent with the hypothesis of an epigenetic origin for this type of somaclonal variant.

5.3.3. RAPD analysis

RAPD analysis was found to be efficient in distinguishing among oil palm clonal lines, but failed to reveal any polymorphism associated with either 'mantled' somaclonal variants or with the overall tissue culture process used to regenerate oil palms (Rival *et al.*, 1997d). Following the scoring of 8,900 RAPD bands, no 'mantled'-related polymorphisms could be detected. This indicates that the frequency of polymorphism in the regenerated material is very low. It may be presumed to be much lower than 0.05%, if compared with the results presented by Munthali *et al.* (1996), who found 3 somaclonal polymorphisms in 5,607 scored RAPD bands obtained from 120 regenerants of sugar beet.

In our experiments, the total length of scored genomic DNA represented approximately 0.04% of the oil palm 2C genome size of 3.8×10^9 bp. Even with the use of more than 380 10-mer primers, the fraction of the genome analysed through the RAPD technique is clearly insufficient for detecting discrete genetic events that could be linked to somaclonal variation in oil palm. Results from the RAPD analysis would, however, be consistent with the hypothesis of an epigenetic origin for the 'mantled' somaclonal variation.

5.3.4. DNA methylation

The role of DNA methylation in the regulation of gene expression (Finnegan *et al.*, 1993) and its implication in somaclonal variation (Brown, 1989; Karp, 1991) has been extensively investigated. Significant changes in the level of methylation of genomic DNA during dedifferentiation (Durante *et al.*, 1982) and during somatic embryogenesis (Loschiavo *et al.*, 1989) have been reported for higher plants. It is therefore likely that changes in DNA methylation levels and/or patterns could be involved in the determination of somaclonal variation in oil palm, as has been demonstrated for maize (Kaeppler and Phillips, 1994).

Levels of global DNA methylation have been estimated after enzymatic hydrolysis of genomic DNA to nucleosides and HPLC quantification of 5-Methyl Cytidine, according to Palmgren *et al.* (1990) and Gehrke *et al.* (1984). Global genomic levels of DNA methylation [(5mdC) / (5mdC+dC)] have been investigated in regenerated oil palms, with the aim of comparing mother palm/regenerants and normal/variant regenerants inside the same clonal line. Global levels of genomic DNA methylation in oil palm reached 25%, in agreement with levels already observed in other plants (Klass and Amasino, 1989). The measured levels of DNA methylation did not discriminate the 'mantled' variants at the adult age. Nevertheless, this approach will be useful for the monitoring of genetic fidelity throughout the *in vitro* culture process, by estimating the role of the various growth regulators involved in the tissue culture process on the methylation patterns of genomic DNA (Loschiavo *et al.*, 1989).

6. Perspectives

6.1. Artificial seeds

The aim of our research in this field is to use oil palm artificial seeds to improve the management, the distribution and the conservation of the clonal material produced through embryogenic suspensions. Our results as a whole show that, at the end of the *in vitro* development, the oil palm somatic embryo does not display the characteristics of a zygotic mature embryo. Thus, further research is needed in order to obtain somatic embryos capable to withstand desiccation and midterm conservation as artificial seeds.

The immature zygotic embryo is considered to be a useful model for studies of the acquisition of tolerance to desiccation. Various osmotica, growth regulators (ABA) and slow desiccation in monitored conditions of hygrometry are currently being tested in order to improve the tolerance of embryos to desiccation. Studies on the impact of these treatments on parameters such as oligosaccharides and storage proteins patterns (Morcillo *et al.*, 1997a) are under way.

6.2. Molecular markers for the 'mantled' somaclonal variation

Long term observations in clonal field trials have shown that the 'mantled' phenotype is unstable (Duval *et al.*, 1997). The fact that almost 50% of sterile palms revert to a normal phenotype after several year's flowering, backs up the hypothesis of an epigenetic mechanism that affects genome expression at the

young age. We now plan to use RFLP in conjunction with oil palm cDNA probes and isoschizomeric restriction enzyme pairs, showing differential sensitivity to the methylation of dC residues (*e.g. Msp*I/*Hpa*II) to investigate the patterns of DNA methylation in oil palm during *in vitro* micropropagation in relation to somaclonal variation.

We are also developing a novel approach based on the analysis of differential genome expression in normal/variant plant material. This approach is centred on techniques available to study differences in the abundance of specific mRNA species between populations. We plan to use the PCR-based Differential Display method (Liang and Pardee, 1992) in order to characterise gene expression in calli and embryoids producing normal and abnormal plants, in the hope of identifying an early marker of the 'mantled' phenotype.

7. Conclusions

Difficulties faced during the implementation of the scaling up of the ORSTOM-CIRAD process of oil palm micropropagation have necessitated the launching of new research programmes. Once the quality control of regenerants can be achieved with molecular markers at a sufficient level of confidence, oil palm clonal micropropagation through somatic embryogenesis could evolve to a larger commercial scale, by means of powerful and low-cost propagation techniques such as the use of embryogenic cell suspensions.

A coherent network has been established, linking the ORSTOM-CIRAD group to several partners in basic research (Universities and Research Institutes) on the one hand. On the other hand, with key players in the oil palm sector in producing countries (private companies, development agencies and national agricultural research institutes). A very close relationship between Biotechnology and Plant Breeding programmes has also been essential in order to: i) efficiently select the elite material to be propagated, and ii) to assess the propagated material in the producing areas according to statistically designed field experiments.

Results presented in this paper illustrate both the importance of the pilot scale step in the scaling-up strategy and the capacity of this step to stimulate important research programmes, involving a fundamental approach with modern research tools.

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