RESEARCH STRATEGIES AND ADVANCES IN OIL PALM CELL AND TISSUE CULTURE

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Cloning of oil palm, in conjunction with breeding and selection, could lead to rapid increases in yield and improvements in oil quality, as well as vegetative features. Oil palm cannot be cloned by conventional horticultural methods; tissue culture appears to be the only feasible approach. Methods and strategies for large-scale propagation of oil palm are described. Fruit abnormalities seen in some clones are described and their likely causes are discussed in the light of available evidence.

Ancillary methods of embryo culture, cryopreservation and protoplast isolation are reviewed. Their application to oil palm crop improvement is also considered.

INTRODUCTION

The oil palm (*Elaeis guineensis* Jacq.) is one of about a dozen commercial sources of vegetable oils. In an increasingly competitive industry, palm oil has grown within a span of three decades to account for about 17% of the total world production of vegetable oils. This has been possible largely because of the emphasis on research aimed at improvements in yield – yields of the order of five tonnes per hectare per year are quite common in commercial plantings in Malaysia. Research emphasis has traditionally been on breeding and selection and on agronomy – both areas indeed having contributed substantially to the present-day plantation status of oil palm. However, whilst these areas of research continue to provide new avenues for crop improvement, it is being increasingly recognized and accepted that all the possible techniques promised by modern advances (*e.g.* in cellular and molecular biology) must be harnessed to sustain the competitive edge that oil palm has so far enjoyed.

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This paper briefly surveys current research strategies in the general area of cell and tissue culture and explores their prospects and problems, especially with respect to clonal propagation.

CLONAL PROPAGATION

Oil palms for commercial plantings are of the thin-shelled *tenera* genotype and are obtained from crosses between *dura* palms, the fruits of which have a thick shell surrounding the seeds, and *pisifera* palms, the fruits of which are without shells. Seed-derived palms, including those from selected progenies, differ from one another in yield, oil quality and vegetative characteristics. Part of this variation reflects the genetic differences between palms; some of it is due to environmental differences. The variation due to genetic differences could be avoided if oil palm was propagated clonally. More importantly, higher yields and improved oil quality could be obtained by cloning palms possessing the desired characteristics.

Suggestions that yield increases of the order of 30% are feasible through cloning have been made (Corley *et al.*, 1981; Wood, 1986). Clonal propagation also has the advantage that secondary characters such as reduced palm height and superior oil quality found in selected individual palms could be uniformly expressed in all individuals (or ramets) of the respective clones. Selections from the new germplasm collections made by PORIM offer much hope to the industry (*e.g.* of reduced height increment and high oil-to-bunch ratio); also of special interest are hybrids between *E. oleifera* and *E. guineensis* species (with, *e.g.* improved disease resistance and a higher unsaturated fatty acid content in the oil). Whilst breeding and selection will continue to be the mainstream method for crop improvement, it is expected that this could be considerably accelerated by clonal propagation. Indeed large yield increases have been achieved with rubber and cocoa by means of clonal propagation.

The morphology of the oil palm precludes the use of conventional horticultural methods (such as budgrafts or cuttings) for clonal propagation: tissue culture seems the only feasible approach. Methods for this were developed in the mid-seventies (see Paranjothy, 1982 for a review) and several organizations now have the expertise for large-scale clonal propagation of oil palm.

The methods used are all essentially identical and rely on callus initiated from actively growing tissues such as young leaves, roots or inflorescences. Embryoids obtained from callus are the source of polyembryogenic self-propagating cultures (also known as calloid cultures). Shoots derived from these cultures are rooted in auxin media whilst the remaining nodular polyembryoids or calloids are subcultured for further regeneration. Published protocols are summarized by Paranjothy (1982).

Callus initiation and embryogenesis are dependent on age of palms, type of explants and genotypes. Thus explants from younger palms (3-5 years) will give rise to callus (and subsequently embryoids) more readily that explants from older palms (> 5 years). Seed embryos from some genotypes are an excellent source of embryogenic callus. The relative recalcitrance to callus initiation and embryogenesis (in callus) derived from explants from older palms is probably a significant hurdle to an otherwise practical method for large-scale propagation of oil palm.

The expectations of improvements in yield and oil quality through clonal propagation, have been somewhat dampened, however, by observations of fruit and flower abnormalities in some clonal palms (Corley *et. al.*, 1986).

MORPHOLOGY OF THE ABNORMALITIES

The abnormalities in clonal palms arise from aberrations in the development of the individual flowers on both the male and female inflorescences. In the flowers of the female inflorescence, the principal abnormalities are the parthenocarpic development of the gynoecium and the development of the normally vestigial stamens into a mantle of fleshy carpels surrounding the fruit. In the flowers of the male inflorescence, the stamens develop aberrantly into carpel-like structures.

The main consequence of these abnormalities are: a) an inadequate number of fertilized fruits, which fail to sustain development of the bunch to ripeness, and b) absence of pollen in the abnormal male inflorescences.

Parthenocarpic fruits are found in the early bunches of normal seedling palms (see, for example, Williams and Thomas, 1970). This feature, however, does not persist beyond the first few inflorescences, whilst it does persist in abnormal clonal palms.

The first few male inflorescences in *dura* x *pisifera* seedlings tend to be androgynous, with the gynoecium developing into carpellary

structures. This feature is evidently more pronounced in young ramets but transitory, as in seedlings. Of noteworthy interest is the occurrence of this feature at very high frequency in *E. oleifera* x *E. guineesis* hybrids; it is also transitory in expression in most seedling hybrid palms, but persistent in some.

THE INCIDENCE OF ABNORMALITIES

The first detailed report of abnormalities in clonal palms was made by Corley *et al.*, (1986). Three clones (31A, 115E and 90A) were planted in Unipamol Estate beginning in 1981. Both male and female inflorescences in the 1981 plantings of the three clones (all from selected palms) were normal. Abnormalities were evident in the 1982 and 1983 plantings. Corley *et al.* (1986) indicated that about 25% and 90% of the ramets planted in 1982 and 1983 respectively were producing bunches with abnormal fruits.

LIKELY CAUSES OF THE ABNORMALITIES

The likelihood of the abnormalities being caused by heritable changes in the genome can be quite readily evaluated. The mantled fruit character is known in wild populations to occur at a frequency of approximately 1 per 1000 palms and is believed to be a dominant character. Seed progenies of abnormal clonal palms can also be selfed and crossed with normal palms to determine the genetic basis of the abnormality.

Several seedlings developed from open-pollinated mantled palms have now flowered (Paranjothy *et al.*, 1989). Their flowering habit is normal, unlike that of the mantled palms. This observation rules out the possibility of the abnormality in the clones being due to dominant gene effects or maternally transmitted factors. Considering the high frequency of the abnormalities after a few passages through culture, it seems unlikely that the abnormalities are a reflection of recessive gene effects. Confirmation of this will be seen in the progeny of selfings of the abnormal palms.

In vitro methods can also be helpful in studies aimed at understanding the causes of the abnormalities. Thus abnormal palms have been recloned and seedlings regenerated from mantled fruits. These plantlets and seedlings have been field planted and are expected to clarify the causal nature of the abnormalities, *i.e.* whether they can be transmitted meiotically, or mitotically or both.

Besides test crosses, there are numerous standard methods for detecting heritable genetic changes. These include evaluation of isoenzymes, restriction fragment length polymorphisms (RFLP), chromosome number and karyotype (including chromosome banding). No differences in chromosome number or karyotype have been observed to date between normal and abnormal palms and differences in isoenzyme patterns seem to be absent (Tan *et al.*, 1988). RFLP methodology has not revealed any differences between normal and abnormal palms (Cheah *et al.*, 1989). It thus appears that the application of methods which will detect fine differences in the genome will probably be essential, if indeed the abnormalities are caused by heritable changes in DNA.

Cytological studies on oil palm callus, though somewhat limited, do reveal changes in chromosome numbers such as are typically seen in callus cultures of other plants. Oil palm ramets, however, are produced from embryoids or polyembryogenic cultures and chromosome counts of ramets, including abnormal palms have so far been normal (Jones, 1983; Mahani, Unpublished). There are possible reasons, a likely one being that cells with chromosome abnormalities are less totipotent than cells with their normal chromosome complement. The regeneration of normal palms through a second generation of callus redeveloped from ramets supports this view (Wooi, 1984). The cytological studies on ramets are supported by field evaluation of ramets (Jones, 1983). Evaluation of fruit characters (which are known to be highly heritable) certainly does not indicate gross changes in the genome (Corley *et al.*, 1981). Finally, the similarity between ramets and their respective ortets is also suggestive of genome stability (Wooi, 1984).

On the one hand there is evidently stable transmission of the fruit and bunch characters (most of them known to be highly heritable). On the other hand, with continued plantlet production there is a tendency towards feminization, first in the female inflorescences and later in the male inflorescences (see *Table 2* in Corley *et al.*, 1986). Thus with prolonged maintenance of cultures there might possibly be gradual changes in regulatory aspects of developmental processes – first in the already feminized female bunches and later in the male inflorescences.

At least one feature (*i.e.* parthenocarpy) is also found in young normal seedling palms.

Corley *et al.* (1986) observed one clone that was abnormal in having parthenocarpic (but not mantled) fruits. It is quite possible that parthenocarpy (which can be induced in palms by exogenous application of auxins) is the mildest expression of the abnormality. Also noteworthy in this context is that feminization of stamens in the female bunches is greater than that in the male inflorescences, both in frequency of expression and order of expression (*Table 2* in Corley *et al.*, 1986). There is clearly an increase in the intensity of feminization with progressive subculture, with the male inflorescences being feminized generally in the later production batches. Other features seen in abnormal ramets, such as development of stamens into supernumerary carpels, are not seen in young seedling palms.

These abnormalities are suggestive of changes in developmental regulatory mechanisms. Very many genes are involved in developmental processes and the regulation of flower development can be regarded as having been canalized in such a manner that all parts of the flower in the female inflorescence develop along the same lines as in the development of parthenocarpic carpels; likewise stamens in the male inflorescence develop into what are essentialy parthenocarpic carpels.

The expression of axillary apices in oil palm is clearly determined either as male inflorescences or as female bunches. The main apex in oil palm is determined in a manner that ensures its growth as a vegetative apex, whilst the axillary apices are redetermined for reproductive functions. *In vitro* systems seem to interfere with these states of organ determination. One notable feature of oil palm cloning seen *in vitro* is that of plantlets with determinate growth, *i.e.* plantlets in which the vegetative apex starts behaving like an axillary bud. Most of these plantlets with determinate growth reveal some degree of feminization. Rarely, but significantly in the context of this phenomenon, young ramets in the nursery have been observed with mantled fruits. The feminization that one observes in the abnormal palms might therefore reflect determination of the reproductive organs under physiologically juvenile states. One clone with a high proportion of determinate growths indeed has very narrow juvenile leaves (Paranjothy, unpublished). A high frequency of determinate growths has also been observed in clone 31A, known to be severely abnormal (Wooi, private communication).

The abnormalities can thus easily be viewed as an epigenetic phenomenon. Corley *et al.* (1986) have suggested that the underlying mechanism might be one of habituation, a phenomenon in which cells *in vitro* acquire the ability to synthesize auxins or cytokinins (or other organic compounds) to an extent where exogenous supply is no longer required. One feature of the abnormalities, parthenocarpy, can certainly be induced in oil palm with auxins.

The unstable expression of the abnormalities (*e.g.* both normal and abnormal bunches on the same palms) and the observation that clonal palms with abnormal fruits revert to completely normal palms (Durand-Gasselin, Unpublished) further support an epigenetic mechanism.

Studies carried out in PORIM (see also Paranjothy *et al.*, 1989) on determinate growths seen *in vitro* (with morphological features of feminization) have provided some especially useful information. These feminized growths have been observed in about six clones, and their appearance is usually evident after the ninth subculture. Their frequency is high in cultures grown on media containing benzylaminopurine, and other cytokinins. Transfer of cultures to basal media resulted in a significant reduction in the number of determinate growths. Treatment of young shoots *in vitro* with high concentrations of benzylaminopurine resulted in the appearance of carpellary growths, confirming the role of this substance in feminization. These observations point to a causative role for cytokinins in the development of abormalities *in vitro*.

Investigations into the hormonal status in abnormal palms, especially in developing inflorescences, might reveal differences and such studies are being pursued now in several laboratories. *In vitro* studies aimed at determining the factors promoting development of terminal inflorescences, and field studies aimed at reversing the abnormalities, by application of growth substances for example, are also in progress.

EMBRYO CULTURE

Embryos can be easily excised from surface-sterilized oil palm seeds and cultured *in vitro* (see Paranjothy, 1982, for a review). The seedlings grown *in vitro* can be established in soil within a period of two to three months.

One application of embryo culture is for germination of the shell-less seeds from *pisife-*

ra and the thin-shelled seeds from *tenera* palms, which both germinate poorly. Embryo culture is already used routinely in PORIM to rescue embryos from these categories of seeds.

The method also invites evaluation as an alternative to the seeds for transfer of germplasm. Besides aseptic transfer, a potential advantage of this approach is that it would enable sampling of immature fruits during prospection and hence increase the number of palms that could be sampled at a particular site. Though fruits ripen about 20 – 22 weeks after pollination, the embryos are fully developed by 10 – 12 weeks.

GERMPLASM STORAGE AND MANAGEMENT

Currently the storage of oil palm germplasm is entirely restricted to field gene banks. This has the advantage that germplasm is immediately evaluated and available continuously for breeding and selection purposes. However, maintenance of field gene banks over long periods is of course expensive in terms of management and land requirements. Furthermore, there is the risk of losses of palms through diseases and pests.

Seed embryos have been stored in liquid nitrogen without impairment of their ability to germinate (Grout *et al.* 1983). Seeds stored in liquid nitrogen will not germinate normally but embryos excised from seeds stored in liquid nitrogen have been found to germinate normally *in vitro* (Paranjothy *et al.*, 1985). These methods have been adequately and satisfactorily worked out for oil palm, so that their application for long term storage can now be considered seriously. Loss of cultures through human error or equipment failure are potential disadvantages. Against these are the advantages of minimal attention, minimal space requirements and minimal genetic change during storage.

Methods have also been worked out for storage of embryoid cultures derived from somatic tissues (Engelmann *et al.*, 1987; Tarmizi and Paranjothy, 1988). The regeneration of plantlets from these cultures may be a potential asset when there is a need for replicates of desired genotypes. The risk of genetic change during culture is obviously a potential disadvantage. Besides its use for germplasm, cryopreservation of somatic embryos would also be expected to find application in maintenance of cultures for clonal propagation.

It will be evident from the foregoing that the various *in vitro* methods have potential application in storage and management of germplasm. It is of paramount importance that these methods be proven safe from the viewpoint of retention of genome integrity. Extensive field trials of plantlets derived from cryopreserved seed embryos and of plantlets derived from cryopreserved somatic embryos are already in progress. Meanwhile culture protocols have been modified to exclude growth substances as far as possible. Thus it is possible to germinate excised embryos in media without growth substances merely by replacing agar with Gelrite (Paranjothy and Rohani, Unpublished).

Besides improved protocols and field evaluation it is also necessary to work out the logistics of aseptic germplasm transfer: it may be necessary to culture embryos at an intermediate station and transport aseptic cultures to their final destination. In principle this appears simple. In practice, however, a great deal of planning and organization will be required. Finally the costs involved must also be taken into consideration.

ANTHER CULTURE

The applications of homozygous diploids in breeding, especially for an outbreeding perennial like oil palm, are potentially great. Attempts at inducing development of oil palm haploids through anther culture have so far been disappointing. It is quite likely that generation of haploids will be low in frequency owing to the accumulation of recessive lethals through outbreeding. Development of multicellular bodies and embryoids from coconut pollen has been reported (Tuyen and Guzman 1983; Monfort, 1985) but haploid plantlets have yet to be produced.

PROTOPLASTS

The isolation of protoplasts from oil palm was first reported by Vouyouklis (1981) using leaves and roots as source material. Bass and Hughes (1984) described the isolation and culture of protoplasts from oil palm suspension cultures. The protoplasts were cultured using a 'nurse culture' of oil palm cells. At PORIM, protoplasts are routinely isolated from a range of oil palm tissues. Protoplasts prepared from leaves and inflorescences have been found to regenerate cell walls and undergo limited cell division to form microcolonies (Ismail and Ali, Unpublished; Sambanthamurthi, Unpublished).

Sambanthamurthi *et al.* (1987) described the isolation of protoplasts from embryogenic cultures and the mesocarp of oil palm. The protoplasts isolated were used for studying fatty acid biosynthesis. Protoplasts incubated with ^{14}C-labelled acetate synthesized a different pattern of fatty acids from tissue slices. While tissue slices produced radiolabelled fatty acids resembling the endogenous fatty acids, protoplasts produced large amounts of palmitoleic and vaccenic acids. The results show that the biosynthetic pathway of fatty acids in normal tissue (16:0 to 18:0 to 18:1Δ9) has been altered in protoplasts (16:0 to 16:1 to 18: 1Δ11). The protoplasts thus provide an interesting system with which to investigate desaturation in the oil palm and its regulation.

BIOCHEMICAL STUDIES

Plant cell cultures provide useful and easily manipulated systems for carrying out biochemical studies. The cells can be grown reproducibly and uniformly in defined media, providing control over cell type and growth stage. Administration of precursors (labelled or otherwise) is relatively easy to control, and subsequent processing is greatly facilitated. Furthermore, the effects of environmental conditions such as temperature, illumination and aeration, and of inhibitors and growth factors can be easily investigated.

Oil palm cultures have been used to investigate the biochemical changes that occur during embryogenesis. Turnham and Northcote (1982) showed that embryogenesis in oil palm is characterized by the deposition of lipids. This deposition of lipids correlated with changes in the activity of acetyl CoA carboxylase, an enzyme which catalyses the first committed step in fatty acid biosynthesis. Acetyl CoA carboxylase activity could thus act as a marker of somatic embryogenesis.

Radioactive incorporation studies have also been carried out to monitor the synthesis of lipids during embryogenesis (Turnham and Northcote, 1984). The synthesis of both triacylglycerols and polar lipids increased during embryoid formation. A rapid increase in the synthesis of polar lipids was observed just before the embryoids became visible and corresponded with the rapid rate of cell division.

CONCLUSION

Oil palm can be propagated clonally on a large-scale using tissue culture methods. This is fortunate, since horticultural methods are inapplicable to oil palm. Successful commercialization of oil palm cloning will be dependent on methods for detecting the rather unusual epigenetic floral abnormalities that have been encountered in some clones. Biochemical markers such as isoenzymes or hormone assays might be necessary to screen out potentially abnormal cultures. In addition to screening for epigenetic changes, routine screening for genetic changes, perhaps using restriction fragment length polymorphisms, might be an additional necessity.

Alternative strategies could be adopted, *e.g.* direct embryogenesis or (limited) multiplication of selected parents for seed production. *Dura* and *pisifera* clones have been established successfully for the latter purpose but direct embryogenesis is a phenomenon that at present is restricted to leaf explants derived from palms less than three years old.

Ancillary methods such as embryo culture and cryopreservation are now sufficiently developed for their evaluation in applications such as germplasm transfer and storage.

It is of paramount importance that the very empirical approaches employed in tissue culture be supported by basic studies. The reliance on such empirical approaches to embryogenesis and the problem of abnormalities perhaps highlight the need for basic research in oil palm, even in areas of molecular biology and biochemistry which have been confined in the past to model systems.

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REFERENCES

BASS, A and HUGHES, W (1984). Conditions for isolation and regeneration of viable protoplasts of oil palm (*Elaeis guineensis*). *Plant Cell Reports, 3,* 169-171.

CHEAH, S C; SITI NOR AKMAR ABD; OOI, LESLIE C L; RAHIMAH ABD RAHMAN and MARIA MADON (1989). DNA marker techniques for the oil palm. *Proceedings of the PORIM International Palm Oil Development Conference,* (In press).

CORLEY, R H V; LEE, C H; LAW, I H and

WONG, C Y (1986). Abnormal flower development in oil palm clones. *Planter, 62,* 233-240.

CORLEY, R H V; WONG, C Y; WOOI, K C and JONES, L H (1981). Early results from the first oil palm clone trials. In: *Oil Palm in Agriculture in the Eighties.* E Pushparajah and P S Chew, (eds). Incorporated Society of Planters, Kuala Lumpur, 1981 p. 173.

ENGELMANN, F; DUVAL Y, and PANNETIER, C (1987). Use of cryopreservation for setting up a bank of oil palm somatic embryos. *Proc. of 1987 Inter. Oil Palm/Palm Oil Conf. – Agriculture,* Kuala Lumpur, 1987, p. 648.

GROUT, B W W; SHELTON, K and PRITCHARD, H W (1983). Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for genetic conservation. *Ann. Bot., 52,* 381-384.

JONES, L H (1983). The oil palm and its clonal propagation by tissue culture. *Biologist, 30 (4),* 181-188.

MONFORT, S (1985). Androgenesis of coconut: embryos from anther culture. *Zeitchrift fur Pflanzenziichtung 94 (3),* 251-254.

PARANJOTHY, K (1984). Oil Palm. In: *Handbook of Plant Cell Culture.* P V Ammirato, D A Evans, W R Sharp and Y. Yamada, (eds.) Macmillan Publishing Co., York, pp. 591-605.

PARANJOTHY, K; ROHANI O, and HASHIM, A H (1985). Oil palm germplasm conservation: The possible use of *in vitro* methods. *Proc. of Inter. Workshop on Oil Palm Germplasm and Utilization.* Kuala Lumpur, 1985. p. 202.

PARANJOTHY, K; ROHANI, O; TARMIZI, A H; TAN, C S and TAN, C C (1989). Current status and stategies of oil palm tissue culture research. *Proceedings of the PORIM International Palm Oil Development Conference.* (In press).

SAMBANTHAMURTHI, R; OO, K C and ONG, A S H (1987). Lipid metabolism in oil palm (*Elaeis guineensis* and *Elaeis oleifera)* protoplasts. *Plant Science 51,* 97-103.

TAN, C S; PARANJOTHY, K and TARMIZI, A H (1988). Variation in esterase isoenzymes in oil palm cultures. Paper presented at 7th FAOB Symposium, Kuala Lumpur, 1988.

TARMIZI, A H and PARANJOTHY, K (1988). *In vitro* storage of oil palm polyembroygenic cultures. *Proceedings of Simposium Biologi Kebangsaan Ke-III.* (In press).

TURNHAM, E and NORTHCOTE, D H (1982). The use of acetyl-CoA carboxylase activity and changes in wall composition as measures of embryogenesis in tissue culture of oil palm (*Elaeis guineensis*). *Biochem. J., 208,* 323-332.

TURNHAM, E and NORTHCOTE, D H 1984). The incorporation of [1 – ^{14}C] acetate into lipids during embryogenesis in oil palm tissue cultures. *Phytochemistry, 23,* 35-39.

TUYEN, N T T and GUZMAN, E V (1983). Formation of pollen embryos in cultured anthers of coconut (*Cocos nucifera* L.) *Plant Science Letters, 29,* 81-88.

VOUYOUKLIS, G V (1981). Une methode d' isolement de protoplastes du palmier a huile *Elaeis guineensis* Jacq. *Phyton., 40 (2),* 167-178.

WOOD, B J (1986). *A Brief Guide to Oil Palm Science,* Incorporated Society of Planters, Kuala Lumpur. 39 pp.

WOOI, K C (1984). Palm tissue culture. In: *Micropropagation of selected rootcrops, palms, citrus and ornamental species.* FAO Plant Production Paper, No. 59, FAO, Rome, pp. 89-112.