# Biotechnological Intervention for Oil Palm Improvement

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Tissue culture of oil palm started during 1960s (Stavitsky, 1970; Rabechault *et al*., 1970; Smith and Jones, 1970) as a component of Biotechnology. Main purpose was to mass-multiply the elite palms, which otherwise could not be propagated as genetically similar and homogenous progenies. Apert from propagation of tissue culture, other aspects of oil palm biotechnology *viz.* Genetic engineering or Transformation, Marker assisted breeding started much later. Biotechnological tools are presently being use for resistance breeding also. Different aspects of Biotechnological research for oil palm improvement have been discussed in this article.

**Tissue Culture**

Oil palm is a slow growing cross-pollinated plantation crop. Commercial oil palm varieties are conventionally obtained from crosses between parents selected after progeny-tests. Unlike the common practice with annual plants, it is impossible to produce homozygous parental families, because of the length of oil palm reproduction cycle and strong inbreed depression. Hence, the progeny tested are genetically heterogeneous. Vegetative propagation of true to type elite palm is the only alternative proposition.

Unfortunately, oil palm does not sucker like certain Cocoids and there is no way of propagating it vegetatively except through *in vitro* culture (Baudouin *et al.,* 1995). Basic process of tissue culture is unchanged from the initiation of the work. This involves seven stages : i) Sampling of explant from selected ortet ii) Callus initiation in explants, iii) Embryod formation (embryogenesis) in callus, iv) Embryod maturation and multiplication, v) Shoot regeneration, vi) Rooting, and vii) Transplanting of plants from ex-vitro condition (Rohani *et al*., 2001).

During Mid 1970s success of oil palm tissue culture has been reported (Rabechault and Martin,1976; Jones,1974). Mainly the outstanding elite palms were selected as ortet. The main characters, which were considered to be very important for clonal propagation was high palm oil yield (in terms of both high bunch yield and extraction rate, slow vertical growth and limited girth (Rajanaidu,1987; Soh, 1998), disease resistance like *Fusarium* wilt tolerance and resistance to *Ganoderma* (Rohani *et al*., 2001). Rajanaidu *et al*. (Rajanaidu et a., 1997) suggested the selection of ortet with a FFB yield of > 200km/ palm/year and presently oil palm breeders including those in PORIM aim for an 'oil to bunch' (O/B) of atleast 27% (Rohani *et al*., 2001). Some Nigerian materials showed 10t/ha/year of oil yield with height increment of 20-25 cm in Malaysia with 45-75 cm of commercial material. Other criteria like high iodine value, high kernel content, harvest index, yield stability, tolerance to low nitrogen level are also been proposed for ortet selection (Rajanaidu,1987).

**Choice of explants:** Root, inflorescence or leaf can be used as explants. In case of roots a trench around the palm is made to assure the sampling from the correct palm. Fresh root tips can be repeatedly sample without much

damage to the palm. But in this case contamination rate is very high (up to 60%) since surface sterilization of roots becomes difficult.

In the case of inflorescence, spikelets and the individual flower reside inside the spathe at a very tender stage and hence can be handled without any surface sterilization under the laminar flow.

Leaf explants are sampled usually without damaging the apex meristem and the same palm can be sampled after about two years, when shoot resumes its growth.

Between leaf and inflorescence explants, leaf gives a higher clonability rate (Rajanaidu *et al*., 1997). Leaf calli are also more vigorous and more embryogenic than those derived from roots (Paranjothy, 1987). Most oil palm tissue culture laboratories use leaf explants with only a few using inflorescence and roots (Rohani *et al*., 2001).

**Culture media and conditions:** Culture media usually contains organic salts, vitamins, amino acids, plant growth regulators, sugar, water and a gelling agent. Activated charcoal is essential for good root growth in liquid media. Volume of media, type of vessels varry from laboratory to laboratory.

A typical condition of growth is 24 hours darkness for explant and callus culture; 12:12 hours photoperiod for embryo proliferation, shoot regeneration, shoot development and rooting. Growth temperature is maintained at 28 $\pm 2^0$C (Rohani *et al*., 2001). However, since the oil palm clones are high valued commercial material, the exact protocol with media composition and cultural conditions are maintained as trade secret.

**Time frame:** Once the protocol is standardised, it takes about five years for field plantinmg the clones from the date of collection of explants. A time frame is given in Table 1 (Rohani *et al*., 2001).

**Table 1: Time Frame of various stages in cloning**

| S.No. | Production stage | Average time for each stage (months) | Cumulative time(months) |
|---|---|---|---|
| 1 | Explant to callus | 6 | 6 |
| 2 | Callus to embryoids | 9 | 15 |
| 3 | Polyembryods to shoot | 24 | 39 |
| 4 | Shoot development | 2 | 41 |
| 5 | Rooting/Transplanting/Acclimatization | 4 | 45 |
| 6 | Pre-nursery | 3 | 48 |
| 7 | Field planting | 10 | 58 |

The major work on *in vitro* culture of oil palm were carried out in the Institute Recherche de Huiles et Oleogeneux (IRHO), France; Wye College, University of London, UK; Unilever Plantations and Plant Science Group, UK; Palm Oil Research Institute of Malaysia (PORIM), Malaysia; Marihat Research Station, Indonesia; Nigerian Institute of Oil Palm Research (NIFOR), Nigeria.

In India, first report of *in vitro* regeneration protocol for oil palm was from Bhaba Atomic Research Centre, Bombay (Thomas and Rao, 1985) and later from Central Plantation Crop Research Institute, Kasaragod (Raju at al, 1989; Karun and Sajini 1996). All the cases explants from seedlings were used. Moreover, no report is available on the performance of those clonal plants.

**Abnormalities in the clones**

Though, the proposition of clonal propagation looked very attractive, a major problem reported to be associated with it was the abnormalities of the clones. Since this is tissue culture derived variation, they are called somaclonal variation.

An early clone produced by Rabechault and Martin (1976) was stated to have given mantled fruit and two types of abnormalities mantled fruit and androgynous inflorescence - appeared in Malaysia in three clones in the 1981-83 plantings (Corley *et al.,* 1986). Abnormal palms are often vegetatively normal with abnormalities largely in the reproductive organs. Also the differentiation of primordia into male and female inflorescence is not affected (Paranjothy, 1986). As with the seedlings, the first inflorescences of clonal palms are invariably male. In palms that subsequently produce bunches with mantled parthenocarpic fruits, the early male inflorescences are often andromorphic. From the practical point of view, severe mantling results in bunch failure due to an inadequate number of fertilized fruit to sustain its development to ripeness. Mildly mantled bunches generally ripen normally. The vegetative characteristics of ramets within clones are generally uniform. However, certain vegetative abnormalities have been detected. They include variations in frond length and morphology such as stiff, erect and drooping fronds, *etc.* (Ho and Tan,1990).

Under normal *in vitro* culture, shoots from within a small clump of Polyembryonic culture can show different morphological forms. It is not easy to differentiate between 'normal' and mildly abnormal shoots at the early stage of shoot development. Only distinct abnormally developed shoots can be distinguished and discarded during subculture. Tarmizi (1997) compared the leaf blades of four off-type shoots against those of a 'normal' shoot using a SEM *viz. Succulent shoot, Rigid shoot, Crinkled shoot and Curled shoot.* Another abnormality of *Terminal inflorescence* also occurs in culture. Severely affected shoots have pointed leaves.

Of the different probable causes of abnormalities of the clones, genetic or epigenetic changes due to stress during tissue culture might be the most important. Changes might be transient or temporary in subsequent generation or may be heritable as in the case of somaclonal variation in other crops. However, other factors also might have a significant role as indicated by several workers *viz.* explant source (Evans *et al*., 1984); age of culture (Symillides *et al*., 1995); hormonal factor (McClintock, 1984); habituation (Paranjothy, 1986); genotypic factor (Rao and Donough, 1990) and DNA methylation (Mandal *et al*., 1999).

Mainly two different research approaches have been adopted by the scientists to tackle this problem - i) to develop a protocol by manipulating the composition and type of media which will give rise to only 'true-to-type' plants and ii) early detection of the variants so that culling would be possible before planting in the main field. An integrated approach comprising of both the methods are being adopted in different laboratories. Several reports are available on modification of cultural media and presently some good strategies like setting subculture limits, type of culture (suspension/liquid/ periodic immersion) are available (Rohani *et al*., 2001). Molecular markers, both protein (Marmey *et al*., 1991) and DNA markers (Rival *et al*., 1997) can be used to screen the clonal materials from the cultural stages itself to avoid abnormalities.

**Yield from clonal plantations**

Clone trial results are now superseding these academic arguments. Corley and Law (1997) listed results of clone trials, and found many examples of clones yielding at least 25% more than seedling standards. It must be noted that the CIRAD-CP standard cross has been in use for many years, and the average yield of 'second cycle' selections is 16% above the standard.Thus, the best clones, most of which were selected from 'first cycle' families, are only about 14% better than currently available seedling progenies. The target is constantly moving: by the time a good family has been identified and the best individuals within it have been cloned and tested, a new generation of improved seedling material will be available. Assuming 10% yield improvement per generation of breeding clones that are 30% better than the generation from which they were selected may only out yield the best seedlings by 20% (Corley and Tinker, 2003).

**Clonal seeds**

Primary objective of all the oil palm tissue culture laboratory was to produce superior *tenera* clonal plants at a commercial scale. Somaclonal variations and low rate of embryogenesis made other alternative clonal propagation strategy like uni-clonal or bi-clonal seed production very attractive (Soh, 1986). However, Corley and Startford (1998) reported that the mantled flowering character can be inherited as can other abnormalities and hence the safety of clonal seed is at present uncertain. Time scale for development of clonal seed might be not faster than for *tenera* clones. Therefore, in their view, clonal seed is not very attractive option for an existing plantation. Where it could have advantages is for new developments (like in India). Yield of a clone at one site will not give a good indication of potential elsewhere. Bi-clonal seed, being genetically heterogeneous, would not suffer so severely from this problem.

**Other *in vitro* culture techniques**

Apart from vegetative propagation, there are other potential uses of *in vitro* culture techniques in the crop improvement programme e.g., protoplsts culture, anther culture and embryo culture.

Protoplasts offer an alternative transformation system and it should be easier to get DNA into cells without walls. (Corley and Startford, 1998). Though oil palm protoplasts have already been produced (Vouyouklis, 1981; Bass and Hughes, 1984; Santoso *et al*., 1991; Sambanthamurthi *et al*., 1996), no report is available on regeneration of plantlets from protoplast.

Developing homozygous oil palm requires at least 40 years as eight generations of selfing is needed for this. Anther culture gives a scope of production of haploid from microspores, followed

by doubling of the chromosome number to produce homozygous diploides. Preliminary reports were found on anther (Latif, 1991) and microspore cells (Tirtoboma, 1998) culture of oil palm but no useful outcome is achieved yet. Use of molecular markers offers a combined approach. Marker linked to useful traits could be used for an initial screening of the double haploid population, so that only the more promising palms were progeny tested. This might reduce the scale of testing to manageable proportion (Corley and Startford, 1998)

Besides the techniques and purpose discussed above, developement of stress tolerant plants (mainly abiotic stress) by inducing the stress in the culture media have been tried in many crops. In oil palm embryos were cultured on modified MS medium for 4 8 weeks before being transferred to filter paper bridges immersed in stress inducing liquid media. Different concentration of sodium chloride and Polyethelene glycol were used to induce salt and water stress respectively. It was observed that oil palm plantlets were more tolerant of water stress than salt stress (Alang *et al*., 1991). No field trial report is found on these aspects.

Embryo culture method involves isolation and culture of seed embryo aseptically. One of the application of embryoculture is to rescue seed embryo of *pisifera,* which normally germinate poorly in vivo. The researchers from NRCOP and CPCRI developed a protocol for embryo rescue from *pisifera* (Anitha Karun *et al.* 2001).

**Future prospect of clonal propagation**

Clonal propagation by culture on solid media is now routine in many laboratories. However, results with any given clone remain unpredictable, with embryoid proliferation being the main bottleneck. As Rival (2000) said, 'customer requirements have to fit in with the vagaries of production, which is not satisfactory from a commercial point of view'. This has stimulated interest in alternative methods, but at the same time the realisation that the amount of somaclonal variation may depend on the culture media or conditions has greatly extended the necessary time scale for development of new methods. It is not sufficient to monitor culture growth rates or frequency of embryogenesis; any new method must also be tested through to the stage of flowering in the field, to ascertain that the palms produced are normal.

Culture of a suspension of cells in a liquid medium offers the possibility of automation of the tissue-culture process, eliminating the hand labour required for transfers on solid media, and with synchronised development allowing the possibility of timing production to meet customer needs. Tahardi (1998) described results with periodic immersion of callus in liquid, rather than continuous liquid suspension culture, based on a method described by Teisson and Alvard (1995). Field trials of clones propagated by suspension culture are in progress (Soh *et at.,* 2001). Preliminary data indicate that the risk of abnormal flowering is no greater than with culture on solid media. This result is very promising if costs could be reduced by the use of suspension culture, then the yield increase needed to make clones profitable would be smaller.

There has been some discussion of the production of artificial seeds, but little work published with oil palm. Clone testing takes 7 or 8 years. During that time, the clones must be maintained in some way, so that after testing the best can be propagated commercially. The possibility of cryopreservation has already been

mentioned but a simpler approach is to reclone the best from ramets in the field (Corley and Tinker, 2003).

Commercial planting of clones has started, on a limited scale. At the end of 2000, there was a total of over 10,000 ha of clones planted world-wide. This area is steadily increasing, as more organisations extend their plantings. Recently in India first set of clonal plantlets have been imported by the Godrej Agrovet Pvt. Ltd.

In India, at present there is no laboratory where the research on tissue culture on oil palm is continuing. NRCOP, Pedavegi is well set to start the tissue culture programme very soon keeping oil palm development in the country in view. However, even after successful regeneration from the elite ortet, commercial scale planting would take some time, as the clones need to be evaluated before they go for commercial scale planting.

**Transgenic oil palm**

**Transformation:** The main objective of transformation is to develop transgenic oil palm with desirable foreign gene(s). Desired gene construct is prepared by genetic engineering, which is transferred to some tissues (by direct insertion or by *Agrobaterium* mediated) of the plant by the process called transformation and further developed into a transgenic plant by tissue culture. Tissue culture is therefore essentially involved in this process. However, recently methods are developed, where the transformation is carried out directly in plant (*in-planta* transformation).

In case of oil palm, mostly the transformations reports are available using direct insertion methods with particle bombardment, the 'gene gun' or 'biolistic'. Parveez and co workers have successfully inserted a marker gene into oil palm tissue and regenerated transformed plants (Parveez and Christon, 1998). The next important step will be the insertion of functional gene rather than simple marker (Corley and Tinker, 2003). *Agrobacterium* mediated transformation is the preferred method for dicotyledonous species. However, some success with monocots have been achieved and Chidamasari *et al.* (1998) have transformed cells in oil palm.

**Target genes for transformation:** Possible objectives for transformation of oil palm include both agronomic characteristics, such as disease or pest resistance, and quality traits-particularly oil composition (all of which are also targets for conventional breeding). Among agronomic characters, modification of the enzymes involved in fruit abscission, which are now well understood (Henderson *et al.,* 2001), could be useful in relation to harvesting. Increase in oleic acid content in palm oil is one of the very important character. Soh *et al.* (1994) suggested that oleic acid content might be increased by increasing the activity of 3-ketoacyl ACP synthase II (KAS-II), or using antisense to reduce the activiy of C16-ACP thioesterase, or both. Other possibilities are (Parveez *et al*., 1994 ) increasing steric acid content by decreasing the δ9-desaturase, increasing palmitic acid content by reducing KAS-II activity, and decreasing saturated fatty acid content by increasing the δ9-desaturase activity.

***In vitro* Conservation**

Conservation of genetic diversity is very important today for the purpose of crop improvement in future. Conserving a huge germplasm *ex vitro* in the field is most difficult job, especially in case of oil palm, as it requires

a vast area and other resources. Conserving seed is quite easy and economic where it is possible. Technology for conserving orthodox seeds (which can tolerate desiccation and low temperature) is well developed and can be stored for years under controlled condition without significant loss of viability. Orthodox behaviour of oil palm seeds in terms of desiccation was first reported in 1983 (Grout *et al.,* 1983). However, it is mentioned that seed of oil palm as a whole can not be stored at sub-zero temperature because of the higher moisture content of the embryo than that of whole seed before and after desiccation. Excised embryos stored up to 8 months in liquid nitrogen (at -196°C) remained viable. Though clonal propagation of oil palm is still under speculation, cryo-preservation of oil palm is mainly concentrated on somatic rather than zygotic embryos. This is mainly to conserve the elite germplasm (clonal), which is not likely to be heterogeneous like zygotic embryos.

**Marker assisted breeding**

Molecular markers are being used in many crops, mainly to reveal the variability between the individuals, which is not possible through the traditional means. As in the case of other crops, marker-assisted selection is one of the priority areas of research in oil palm and is having the most important role in biotechnology at present. Though there are large numbers of markers available and some work has already been done with proteins and isozyme markers (Ghesquiere, 1984; Boudin, 1992, Choong *et al*., 1996), the most reliable is DNA marker. DNA markers differ from other marker systems in two major respects: i) any tissue can be examined (e.g. leaf, root, pollen, callus from tissue culture etc.) and many loci can be scored on the same DNA preparation, which itself can be stored indefinitely and ii) the genotype is scored directly rather than through expressed genes (phenotype).

There are essentially two distinct types of DNA markers: random markers and those linked to useful traits. Random systems are relatively easy to develop; they are used for studying genetic diversity or calculating the relatedness for planning crossing programme, conservation and prospection projects. They are also used for 'fingerprinting' to determine the identity of clones or the legitimacy of progenies.

At NRCOP, Pedavegi the work has been initiated on genetic diversity study using randomly amplified polymorphic DNA (RAPD) markers (Mandal *et al.,* 2004). A new and novel method of DNA extraction procedure has been developed by the researchers of this Centre (Jayanthi *et al.,* 2004). Very soon Simple Tandem Microsatellite Sequence (STMS) markers would be employed for genetic diversity study and palm identification.

Finding markers linked to useful traits is more difficult, in that detailed statistical analysis of the segregating population for the trait of interest is required, and a large numbers of markers may have to be tested before linkages are found. The first step is the construction of a linkage map; this allows a set of markers to be identified which gives reasonable coverage of the genome, thus allowing the contribution of specific chromosome regions to be determined.

Development of a shell thickness marker for identification of varieties at the early stage is very important. Mayes *et al*. (1997) reported a RFLP marker and Morentzson *et al* ., 2000 identified two RAPD markers linked to shell thickness, but their use for practical purpose is limited might be because they are not closely linked. Work on shell thickness marker has also been initiated at NRCOP (Mandal and Pillai

2005). Rajinder *et al*. (2001) identified marker linked to carotene content and oleic acid content of the oil. In principle these linkage allows selection at the seedling stage itself. Such linkages are likely to be specified to the population studies, so continuing work is needed using advanced and reliable marker system, is this approach is to be useful for the breeders (Corley and Tinker, 2003).

Resistance breeding is a part of molecular breeding though it is a part of crop protection. Approach is similar to other characters and here disease or pest resistance/tolerance is consider to be controlled by single gene or by quantitative trait loci. A proper mapping population would help in tagging the resistance gene, which is time consuming in oil palm compared to other field crops.

However, Molecular marker assisted breeding is the most promising area under biotechnology for the improvement of oil palm. This can reduce the time of breeding programme considerably; it is more precise and also helps in reducing the requirement of resources like land, labour and capita.

**Indian context**

Oil palm is an introduced crop in India and it has come to the farmer's field only in the last decade. As the potentiality and prospects of oil palm has been recognised gradually by every sector of the people including farmers, common people (as edible oil consumer) and entrepreneurs, research and development on different aspects of oil palm is getting more and more importance in the country. Biotechnology has a big role to play in the oil palm improvement programme. Research work on Biotechnological aspects has already been started and a few results are already described in the relevant section. However, few of the priority areas in the Biotechnological research for oil palm improvement are : i)Development of a fool proof regeneration protocols from elite palms, ii)DNA fingerprinting for genetic diversity study, iv) Linkage and QTL mapping for important traits and their use in marker assisted breeding iv) Standardization of transformation protocol and iv) Finally development of transgenic oil palm with the desirable traits.

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