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Use of Biotechnology for oil palm improvement.

Background paper by L.H. Jones, University of Cambridge

1. INTRODUCTION

In this background paper I shall review the current position relating to the applications of biotechnology to crop improvement in the Oil Palm.

I shall then discuss the relevance of these modern technologies to practical problems in relation to African needs (as I currently perceive them as a complete outsider), and ask which, if any are appropriate.

We can then consider how the relevant technologies might be acquired, how they should be supported, and how they can be integrated into the current practices of oil palm breeding, seed production and plantation management.

2. CURRENT STATE OF THE ART

Applications of biotechnology to crop improvement are almost exclusively concerned with aspects of plant breeding. They range from clonal propagation, using tissue culture techniques, through various manipulations at the cell genetic level, such as the use of protoplasts for cell fusions or manipulation of haploid cells from pollen culture, to the use of recombinant DNA technology for the transfer of specific genes. Molecular biology also provides very powerful tools for the plant breeder in mapping the genome and identifying individual genes of relevance to the breeding programme.

Progress has been rapid in recent years in the use of biotechnology for the breeding of annual crops, such as tobacco, (a favourite research tool because of its ease of manipulation in culture) oilseed rape, soya beans, tomatoes, rice and maize. The two latter species are in the family of monocotyledons, which in general are less amenable than dicotyledons to tissue culture and the ttraditional DNA transformation methods using *Agrobacterium tumefaciens* Ti plasmid as a DNA vector. Oil palm is also a monocot, and as such is likely to be a more difficult subject than the dicotyledonous species. It is also a perennial crop with a long breeding cycle, which results in a long time-scale for any genetic studies.

Clonal propagation:

At the present time techniques are available for clonal propagation of oil palm using tissue culture. In contrast to other tropical crops such as rubber, banana, cassava and many others, there is no traditional method of clonal propagation available, and the advent of the tissue culture method holds out the prospect for palm breeders to select and multiply clones from individual elite palms from the best available progenies. It has been estimated that yield improvements of 30% should be attainable over the mean oil yield of the progeny from which the clonal selections were made(Meunier *et al*, 1990) . Yield improvements of 20% have already been realised from the first clones produced (Corley 1991).

The methods have been the subject of several recent reviews (Jones & Hughes 1989, Paranjothy 1986, Jones 1990, Wooi 1990). There is now no difficulty for any competent tissue culture laboratory to set up cultures of oil palm, and to recover regenerant plants by following the established published procedures. Indeed there are many such labs in Malaysia, Indonesia, Ivory Coast etc. A comprehensive account of Unilever's experience over the past 15 years, since the first clonal oil palm plants were planted in Malaysia, was given by Corley (1991).
It must be noted that although the techniques have improved steadily since the first plants were regenerated in 1974, propagation of oil palm is still slow, uncertain, and requires constant vigilance and selection of competent tissues from the heterogeneous tissue masses that are characteristic of the type of growth obtained. It is not amenable to simple mericloning methods, and discrimination is required at each subculture to reject non-embryogenic material. Thus it is essential that transfer operators are well-trained and able to assess the state of the cultures. As with most monocot cultures, it is necessary to start with actively growing meristematic tissues, either from young leaf-base tissue, from lateral root initials, or from young inflorescences. In each case it seems essential to induce callus formation to break the organisational integrity of the source tissue before cells are able to form somatic embryos or adventitious buds. In the presence of an auxin (NAA or 2,4-D) a low percentage of callusses will produce somatic embryos. The frequency is greatly dependent on genotype, and is improved by optimisation of medium additives. These improvements have been made over a period of 15 years development, mostly in commercial labs which do not publish their results. The early recognition of the proembryogenic cell clusters, and their transfer to media with reduced auxin content is essential to prevent them reverting to callus and subsequently losing regeneration potential. The embryogenic cultures are then capable of continued proliferation over a long period, producing

shoots that can be removed and rooted, and fresh embryoids for further recycling. During this phase there is always the possibility of cultures losing their regeneration potential or undergoing genetic or epigenetic changes, and it is necessary to reisolate fresh cultures regularly to replace ageing culture lines.

The problems of translating the laboratory techniques into a large scale factory process are not trivial. They require particular attention to hygiene and quality control, and constant attention to detail. It is essential to have good biological management with people who understand the nature of the tissue cultures, can identify the competent cells and can recognise the early signs of any deterioration in quality. A well staffed quality-control laboratory is needed to monitor any signs of microbial or insect contamination, to control the quality of the media in use, and to identify and quickly eliminate any sources of contamination or deterioration of the quality of cultures being transferred. A laboratory is also required to initiate cultures of new clones, and to regularly re-isolate competent cultures of established clones.

Commercial development of the propagation of clonal oil palm has been seriously delayed by the appearance of abnormal flowers in some clones that have been subject to large-scale production. (Corley *et al* 1986). Intensive work on this problem over the past few years has so far failed to reveal the cause, although some clues are emerging. At the International Plant Growth Regulator meeting in Amsterdam this year, Besse *et al* (1991) reported depressed cytokinin levels in abnormal flower tissues as compared with normal tissues from the same clone. They also reported that regenerants from nodular calluses were almost invariably normal, while the friable "fast-growing" calluses gave rise to abnormal palms. Unilever experience has not been so clear-cut: there have certainly been some normal clones from fast growing friable cultures, and abnormals from the nodular types. Over a period of time the frequency of abnormal ramets developed from nodular polyembryogenic cultures increased, although there was no evidence of friable callus (Corley 1991). In the latter cases it is always possible that some FGC (even if only a few cells) developed on the nodular tissues and gave rise to the abnormal embryoids. There is some evidence for a genetic basis of the problem: cultures from some progenies are more prone to produce abnormal flowers than others. Genetically mantled palms occur naturally at a low frequency and in these cases the condition appears to be heritable, and was reported by Beinaert and Vanderweyen (1941) to be due to a dominant gene. Seeds from mildly affected bunches of abnormal palms from tissue culture (still capable of setting seed), subject to open pollination, have been planted and have now

flowered. Some of the progeny were mantled, but the distribution did not suggest the operation of a simple Mendelian dominant gene. (Rao and Donough,1990) . Controlled crosses have been made, but the plants have not yet flowered. In addition, some of the previously abnormal palms have reverted to normal, or at least the frequency of abnormal flowers on the inflorescences is declining. Use of genetic probes has so far failed to reveal any consistent changes in DNA structure (Cheah *et al* 1991), but there is a growing suspicion that there may be changes in the mitochondrial DNA. This might not affect all the mitochondria in the tissues, and hence the abnormality could vary in its level of expression. Reversion to normal could then be explained by rejection of the abnormal mitochondrial population and its replacement by normal mitochondria, perhaps as a result of differential multiplication rates.

Clearly there has been some semi-heritable change in gene expression relating to flower development, resulting in the production of extra carpels from the stamen primordia. This change has been induced in the culture stages. Unilever has examined a wide range of culture conditions, and the Company is now confident that the problem can be avoided by judicious selection of "resistant" ortets, by use of low-risk media, and by limiting the number of transfer cycles. The IRHO group are also confident that provided friable "fast growing callus" is avoided, and only the nodular embryogenic cultures are used, then there is little risk of abnormality developing. Both groups are now engaged in extensive field trials, and are optimistic that large scale production of clonal oil palms can soon be resumed.

Further progress in the development of tissue culture methods was reported by de Touchet, Duval and Pannetier (1990). They successfully obtained an embryogenic suspension culture which could be continuously multiplied in the presence of 2,4-D, but which could be plated out to give rise to individual plants from single somatic embryos. This now offers the prospect of developing oil palm suspension cultures suitable for large scale propagation in a fermentor system, much as carrot and alfalfa cultures have been handled previously. Since their presentation in 1990, the work has progressed to the isolation of suspension cultures of a number of embryogenic lines of different genotypes, and the plating conditions are being optimised to improve the yield of single plants. Work is in progress to develop encapsulation methods to aid automated handling of the individual somatic embryos. Success depended on good careful cytological studies of early stages of embryogenesis and the recognition of the embryogenically competent cell clusters at an early stage. (de Touchet, personal communication). If it is possible to develop these methods to a

commercial scale, the production costs will be dramatically reduced, and almost eliminate the need for rows of transfer hoods (and their operators) in the production unit.

There may be dangers in such a process. Clones produced so far have been derived from relatively few embryogenic events and, apart from the flowering abnormality have proven extremely uniform, with little or no evidence of somaclonal variation (Wooi *et al* 1982). Large scale production from single cells, each giving rise to individual somatic embryos, may result in a hitherto unseen number of somaclonal variants. The French group are currently field testing clones derived from the suspension cultures for uniformity.

Cell Genetics:

a) Protoplasts. : The successful formation of calluses from oil palm protoplasts was reported by Bass and Hughes in 1984 using a nurse culture technique. No regenerant plants were obtained, but there is now a prospect of producing protoplasts from the embryogenic suspension cultures of de Touchet *et al* which might regenerate plants freely. Sambanthamurthi, Oo and Ong (1987) obtained metabolically active protoplasts from embryogenic cultures, but did not attempt to subculture them. Protoplasts from such a system could be used for either for protoplast fusion experiments (eg interspecific hybridisations between *E. guineensis and E. oleifera*, or other palms) or for DNA transformation work.

b) Haploids: Little progress has been reported on culture of oil palm anthers or immature pollen. Odewale (1983) reported callus formation from anther culture of oil palm, but I have not seen the thesis, and do not know whether the cultures originated from microspores or anther wall, nor whether they were haploid. Work is in progress at PORIM, Malaysia, but again, I do not know the present state of the work.

Molecular Biology:

Transformation of oil palm cells with foreign DNA has not been reported. The essential pre-requisites of an efficient plant regeneration system from tissue culture, and a method of transformation, are still lacking. It is unlikely that oil palm, being a monocot, will be transformed by the *Agrobacterium tumefaciens* plasmids, but there are several other promising approaches including the Biolistic approach using direct DNA injection with DNA-coated particles shot into competent cells. Although now popular, most transformants are transient and there are only limited

reports of successful permanent integration of functional DNA using this technique in any species. Other methods include electroporation, and PEG mediated DNA transfer. The advent of the embryogenic suspension cultures from de Touchet *et al.* (*loc.cit*) may make these approaches possible. It would be important to determine whether resulting somatic embryos were derived from single transformed cells. Cells already at the multicelluar proembryo stage would give rise to chimaeras, and transformed cells may not enter the germ line. In any event it would be essential to evaluate the progeny for stable integration of the foreign DNA, and with the long breeding cycle this would be a very long term programme.

DNA transformation has been used in several crop species to transfer herbicide resistance , resistance to insect pest damage (e.g. using *Bacillus thuringiensis* toxin genes), and to incorporate antiviral genes, either for virus coat protein or anti-sense viral RNA. These have proven effective in protecting against viral infection. Fruit quality has been successfully modified in tomato breeding by the use of anti-sense RNA (Smith *et al.* 1990). (Incidentally the number of authors on this paper is indicative of the size of research resource required to implement effective molecular biology work). The number of successful transformations with commercial applications is still limited, but increasing rapidly, and transformed plants are now in field trials in several countries. Undoubtedly the techniques will improve rapidly and the number of applications will increase exponentially over the next few years.

Molecular probes:

Molecular biology has provided very powerful tools for probing and mapping the genomes of crop plants. Restriction Fragment Polymorphisms (RFLPs) enable different genotypes to be distinguished and genetic maps and linkage groups to be established. The recent introduction of the polymerase chain reaction (PCR) for rapid amplification of minute traces of DNA has provided an alternative method which has reduced the time, cost and sample sizes required for these procedures, which can now be done without the need for radioactive probes.(Arnheim *et al.* 1990). However, the very high sensitivity of this method requires extreme care to avoid contamination with extraneous DNA, and results should always be checked with conventional Southern blot analysis before unusual DNA patterns are accepted as coming unequivocally from the sample. At the recent International Oil Palm Conference at PORIM (1991), progress in the diagnostic use of RFLPs in oil palm was reported by Cheah *et al* (1991), and by Mayes and Jack (1991). The technique is now in routine use for clonal

typing and checking the identity of clonal ramets with the original ortet. Evidence obtained so far using the probes available suggests there is very little somaclonal variation in oil palm cultures compared with other species, such as potato. It must be noted however that there may be changes in DNA sequences which are not cut by the restriction enzymes used and do not result in changes in the visible fragment sizes. At Plant Breeding International (Cambridge) a start has been made in constructing an RFLP map of the oil palm genome for use in the Unilever oil palm breeding programme.

3. RELEVANCE OF THE TECHNIQUES:

Relevant technologies are ones which will confer significant benefits to the grower, consumer and/or processor. In the end these can all be reduced to a cost-benefit equation. It is not essential to replace old techniques with modern ones just because they are new and exciting. They must be shown to be worthwhile in terms of cost and productivity. This type of research is expensive and long-term. Evaluation of the benefits of research must be costed to allow for recovery of the research and development costs on a proper accounting basis, for example using discounted cash-flow analysis. What benefits might have been obtained if that money had been spent on other programmes, or even left in the bank? In many cases it will be pointless to introduce improved planting material (at higher cost) when the limitations to productivity lie in problems of soil fertility and agronomic management.

There is no doubt that propagation of clonal palms by tissue culture can result in significant yield improvements over conventional seedling progenies. It is also possible to select clones with resistance to *Fusarium oxysporum*. (Corley 1991).
The application of fermentor techniques and automated handling of the young regenerant plants, when available, will significantly reduce the cost of production of the clonal plants, although with high initial capital costs, and the need for high level technical skills for maintenance and back-up. In order to be effective clonal propagation must be coupled to an active palm breeding program with a wide selection of germplasm and well recorded individual palms in elite progenies. The selection procedures must be based on sound physiological criteria and selection made on the basis of characters known to be heritable (otherwise the selected palms may have had good performance simply because of their growing in a favoured environment, *e.g* good nutrition, optimal water, low competition etc. Such palms would prove to be no better than average when

propagated as a clone). Because the selection criteria are uncertain, and many complex factors interact to determine individual palm performance, it is also essential to test clones extensively in the areas where they will be used (genotype X environment interactions have been shown to be important (Corley *et al* 1988)), and thorough testing is also required to ensure that there are no abnormalities of flowering, or any extensive somaclonal variation. This is particularly important when any changes are made to the tissue culture procedures.

The prospect of significant improvements from protoplast fusions or other cell manipulations are more remote. Most of the examples from the more easily manipulated species are biological curiosities rather than useful sources of new variation for the plant breeder. While there are undoubtedly characteristics in other palms, for instance differences in oil quality, that might be transferred by somatic hybridisations, the chances of maintaining fertility and high yield, without the introduction of other adverse characters is very low. Even if the hybridisation and plant regeneration steps led to fertile offspring, a long back-cross programme would almost inevitably be required to establish the new character in a useful genetic background.

Similarly I am doubtful of the value of haploid breeding in oil palm. With a highly heterozygous outbreeding species it would be expected that the vast majority of haploid cells would be carry many deleterious recessive genes. Thus the regeneration rate may well be very low, and the vast majority of regenerants would be weak. All would be either dura or pisifera, and their value as parents could only be tested by making large numbers of dura X pisifera crosses and testing the progeny. By this means it might be possible to find parents with complementary genes which would give good tenera progeny, but the commitment of resources in field trials and individual palm recording over many years would be enormous. The benefit would be to have truly homozygous parents for production of uniform F1 tenera progenies. The same result, of phenotypically and genetically uniform planting material, can be achieved by cloning the best individuals from segregating progenies.

In the oil palm, which is a highly heterozygous outbreeding species, there is plenty of variation available within the existing germ-plasm, and this can be most efficiently exploited by recombination using classical breeding techniques combined with clonal propagation of the best individuals within the segregating progenies. Conventionally plant breeding has sought to produce uniform seed populations from a relatively narrow genetic base, and the introgression of wild-type germ plasm has been limited. The

ability to produce uniform clones from elite individuals within highly variable segregating progenies allows the breeders to explore a much wider source of germ-plasm than hitherto. (Hardon *et al.*, 1987).

The majority of characteristics of interest to the palm breeder such as yield, drought tolerance, or disease resistance are either controlled by many genes or by genetic systems that are not yet understood. The current state of gene trasnsfer technology , even in the species where it is possible, is still confined to addition of single genes, either constitutively expressed or possibly with developmentally controlled organ-specific regulators. The successful programmes have been based on a thorough understanding of the underlying biochemistry of the enzyme systems in the pathway which is to be modified by the introduced gene. It is difficult to identify any at present which would confer sufficient benefit to the oil palm grower to warrant the cost of a genetic manipulation programme. There are no known virus diseases, the majority of insect pests are easily controlled, and herbicide tolerance is of little interest except perhaps in the early seedling establishment phase, where again conventional management is simple and effective.

In his wide-ranging paper to the recent International Oil Palm Conference, Davidson (1991) outlined several characteristics which would improve the crop by reducing harvesting costs and harvesting losses and making the introduction of mechanical harvesting a practical option. These include better control over sex-ratio, uniform bunch ripening, and control over fruit abscission, longer bunch stalks for ease of harvesting, and better indicators of bunch ripeness. Some of these characters may be amenable to genetic engineering , but first the relevant genes will have to be identified, the biochemical pathways they control understood, and methods found to introduce the appropriate genes under proper control in a stably integrated way. A start has been made by Osborne (1991) in understanding the enzymic control of the fruit abscission process in the oil palm. This knowledge could quickly lead to identification of the specific genes involved, and hence to their ultimate modification. In this instance, when the objective would be to interfere with a natural process by inhibiting the activity of enzymes causing fruit abscission, the use of anti-sense genes may be effective. The question must still be asked; is the advantage to be gained worth the investment required to bring about the desired improvement?

To summarise this section, in my opinion the relevant technologies are firstly clonal propagation by tissue culture, coupled to an active conventional palm breeding programme and efficient clone selection and

evaluation. Secondly the use of DNA diagnostic procedures (RFLPs PCR) again coupled to the conventional breeding programme and used to develop an understanding of oil palm genetics. We have as yet no linkage maps, very few identified genes and even the chromosome cytology is not well documented. The 32 chromosomes are small and difficult to distinguish, and not easily amenable to karyotyping. The diagnostic techniques are also proving valuable in evaluation of cloned ramets and identification with their ortets.

4. ACQUISITION OF THE TECHNOLOGIES

In my view there is little point in attempting to set up independently a large scale tissue culture cloning operation for oil palm.

No commercial company would contemplate developing, in house, from scratch, a copy of an existing technology. It is far quicker and much more efficient (and therefore cheaper) to buy it in from someone who has already developed it. There is no point in struggling to overcome the problems of scale-up and management when these have already been solved. In most cases, companies developing the new processes will either seek to protect them by patents, or by refusing to divulge the technical details. This is understandable when the large investments and long time-scales involved in developing the technologies are considered. Naturally companies will only indulge in such programmes if they know they can both recover their development costs and subsequently make a profit. It must be remembered that the object of patents is to encourage the use of patented processes, not to inhibit it, and once a patent has been issued it should be possible to negotiate a licence to use the process. This is universal practice in most branches of manufacturing technology. Indeed it is probably not worth-while to attempt to set up a separate rival operation, and the modern approach is more and more to contract work out wherever possible. In that way the risks are borne by the contractor, not the customer. There is no requirement for large scale capital investment, no long term commitments, and no problems in maintaining quality. As new techniques are introduced you can benefit from reduced unit prices. There are now numerous tissue culture laboratories with the relevant expertise and multiplication capacity anxious to compete for work. It is therefore possible to negotiate financially competitive contracts for propagation of selected germ-plasm, and if the contractor does not deliver, to put your custom elsewhere. Control over the germ-plasm can be secured by the use of the DNA fingerprinting techniques to ensure there is no risk of plants being transferred to competitors. In any case no reputable propagators would risk the loss of custom consequent upon loss

of confidence in the security of material contracted to them. The important resource is the germ-plasm, and the real effort should be concentrated on obtaining the best genetic material for multiplication, and on its rigourous field testing and selection. That then becomes a highly marketable resource, but will have to prove its superiority to rival sources in the market place. West Africa has the great advantage of being the natural centre of origin of the oil palm. There is a comprehensive genetic collection of both wild and highly developed breeding lines, and a long tradition of oil palm research. Much of the data required for selection of parents for production of progenies suitable for cloning is already in the archives.

Growers and processors want to be able to produce palm oil that will compete in price on the world market where there are steadily increasing supplies available from South-East Asia and South America. The African countries are in a strong position to introduce new genetic variation, including disease resistance (e.g. to *Fusarium* wilt) and to compete strongly with SE Asian material derived from the relatively narrow base of the Deli Duras.

The DNA technology on the other hand, for clonal moritoring and for oil palm genetics to aid the breeding programmes, should be available directly to the palm breeders. To this end it is necessary to acquire the latest techniques, and, because in this field progress is so rapid, there must be regular contact with other molecular biology labs. Regular exchange of people, attendance at international meetings and training workshops will help to maintain an awareness of current methods. The methods in molecular biology are relatively simple, but not cheap. It is necessary to acquire a collection of probes and restriction enzymes, and there are continuous improvements in the methodology rendering equipment out of date within a very short time. Although the preparation of DNA gels is easy, the most important requirement is for experienced workers who can interpret the results and safeguard against the ever-present risk of artifacts. It is also important that the palm breeders are aware of the power of the new technologies, are eager to use them in their work, and can integrate their programmes with the molecular biologists. Plant breeding has traditionally been an empirical art, not necessarily requiring much more than basic genetics. The new techniques require a thorough genetic understanding to be linked to the new molecular information. The implementation of an effective dialogue between molecular biologists, with their highly specialised and largely impenetrable jargon of cryptic neologisms and the plant breeders traditionally reliant on field recording and simple analysis of components of yield requires education on boh sides.

5. USE AND MANAGEMENT OF THE TECHNOLOGY

Having acquired the technology it is essential to apply it to economic advantage and to maintain it with continual updating, and this applies to any biotechnological process which might be adopted. The clonal methods will continue to improve, the factors causing the flowering abnormality will be discovered and evidence of the levels of somaclonal variation will continue to accumulate. This knowledge will determine the ultimate usefulness of oil palm clones. It is important for palm breeders to be in a position to assess the value of the techniques available and to apply them where there are advantages in so doing. Similarly recombinant DNA technology will continue to develop rapidly, and again a constant awareness of the current developments is essential. On the other hand there is little point in trying to develop the methodology for oil palm when there is so little knowledge of oil palm genetics, or of the biochemistry involved in determining the characters which it might be useful to modify. These must be the priority areas for work to provide the background which will make it possible to apply the new technologies as they become available.

The use of clonal oil palm is still in its infancy, and there are many ideas and opinions on how clones should be managed. Optimal planting densities differ between clones (Corley and Donough 1990), particularly if palms with high bunch index are selected with relatively small leaf canopies and short trunks. It is not clear how many clones should be used in a given planting. Is it safe to plant large monoclonal blocks or would it be better to interplant a clonal mixture? Perhaps 2 hectare blocks of maybe 6 clones would provide security against catastrophic failure of any individual clone. It would be easy to clear and replant the affected blocks, where it is not possible to effectively replace individual palms in a mixed planting. Clones have been found to have relatively closely synchronised sexual cycles. If a whole block is in a male phase there is no need for harvesters to visit that block until it re-enters a female cycle. These are only some of the questions to be answered in optimising the use of clonal material, and many years of field trials and recording will be nececessary.

An alternative use of clonal material is for the limited propagation of dura mother palms for seed nurseries producing "clonal seed": in other words to enhance the production of seeds of what are effectively single progenies of proven parents. We know that limited production of clones will have very low risk of any abnormalities, and such seed nurseries will produce high quality conventional seed which can be planted with confidence until such time as the uncertainties concerning large scale tissue culture propagation are resolved.

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