High Efficiency Vegetative Amplification- A New Oil Palm Improvement System

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Abstract

Dami Oil Palm Research Station (OPRS) in West New Britain Province of Papua New Guinea is the research and development agency of New Britain Palm Oil Ltd (NBPOL), the largest sustainable palm oil producer in that country. Dami seed from NBPOL is exported to many countries and enjoys a reputation for improved palm oil production.

The OPRS breeding programme has identified individual progeny tested families that deliver CPO yields of over 9 tonnes per hectare per annum, a gain of 50% over the company's average plantation yield of 6 tonne/ha/annum. Individual trees within these trials promise even higher potential gains if they could be cloned successfully.

We report here the results of new techniques for the induction and proliferation of embryogenic tissue on immature inflorescence secondary explants from elite *Tenera* palms. Within 12 weeks of collecting inflorescences from 8 different ortets, the best treatment produced compact proembryogeneic masses (CPEM) on 97.4% of the spikelets from males and 90.5% of the female. All ortets produced embryogenic tissue which covered an average of 32.4% of the surface area of each male and 44.9% of each female secondary explant (subdivided individual spikelets).

CPEM in shaker culture produced small tissue masses that formed clusters of small, white somatic embryos (SE) when plated on semi-solid medium. The SE clusters could be stored, or germinated after only 2 weeks on germination medium to produce first radicles then epicotyls on each embryo. In initial trials, 5 germinated embryos were produced from each millilitre of shaker culture. Plants are now ready for transfer to the rametry.

We propose to adopt the concept of 'vegetative amplification' (also known as 'family forestry') developed in New Zealand pine forestry. High-efficiency somatic embryogenesis will be used to 'amplify' the best ortets from our advanced breeding progeny trials to provide families of ramets for planting oil palm plantations. The genetic gain from use of our best families will thus be 'amplified' across a larger land area than possible through using seedlings from the same families.

Although we are not the first to report a genuine somatic embryogenesis process for oil palm, we believe that this is the first report of production of hundreds of oil palm

plants where radicle emergence is seen before coleoptile emergence from singulated embryos on a scale that promises to be readily adapted to industry requirements for cloned palms.

Background

From the first demonstrations of propagation of oil palm through tissue culture in the 1970's, cloning of elite palms has been promoted as a powerful tool for increasing the productivity of plantations. Cloning was offered as a more efficient option than the long breeding cycles of recurrent selection and tree breeding. The reality has been somewhat different – the high costs, low propagation efficiencies, and the problems of flower and fruit abnormalities have resulted in tissue culture planting stock making up only 2-3% of the annual Malaysian planting programmes. In the meantime breeding studies in the industry have begun to deliver solid genetic gain.

New Britain Palm Oil Limited (NBPOL) has 75 thousand hectares of plantations in Papua New Guinea and the Solomon Islands. NBPOL has an advanced palm breeding programme, a tissue culture laboratory, and a commercial seed production unit at the Dami Oil Palm Research Station (Dami OPRS) in West New Britain Province. The original NBPOL tissue culture protocols have produced clones for use as parents in commercial seed gardens and ramets for field trials (Vovola and Lord 2004).

The Dami breeding programme was described by Dumortier and co-workers (Dumortier et al, 2006) Dami breeding families are now available that have delivered progeny-tested family averages of more than 9 tonnes of Crude Palm Oil (CPO) per hectare per annum - a gain of 50% over the mean NBPOL estate average of 6 tonnes CPO / hectare/annum.

Limitations of current tissue culture protocols

Although oil palm is arguably the first major tree crop to be clonally propagated through tissue culture beginning in the 1970's, the world-wide use of this technology is probably less than 2 percent of total annual planting. The first and most notorious constraint is that of fruit and flower abnormalities including 'mantled' fruit. Reviews of this problem have suggested solutions such as limiting the duration of the 'embryoid' culture phase (Eeuwens et al, 2002) and avoidance of 'fast-growing' callus. The MPOB liquid suspension technology is reported to have reduced the incidence of mantling to acceptable levels in an industrial oil palm tissue culture laboratory in Malaysia (Wong et al, 1997; Soh et al 2001).

Early in the development of oil palm tissue culture technology, long time intervals of 3-5 years between ortet sampling and ramet deployment were cited as constraints to industrial utility of tissue culture in general plantation management. In more recent years the long time interval has been reduced and the recent emergence of large tissue culture facilities producing in excess of 1 million tissue cultured plants per annum is observed (Soh et al 2001).

The percentage of formation of genuine embryogenic tissue from callus produced on the primary explants (the original explants sampled from the ortet) is cited as one of the major present constraints to cost-effective oil palm cloning. Soh and co-workers

have also pointed out some of the dangers of the uncritical acceptance by managers of the notion that clones are 'inherently' superior to seedlings and rightly draw attention to the need for prolonged field testing, and integration of clonal selection with ongoing breeding programmes.

Individual ramet cost is perhaps the biggest constraint to wide adoption of cloned oil palm by the industry. This is a topic that has not attracted much attention or analysis in the published oil palm literature. It is apparent from articles in the Malaysian news media that the industry is currently paying prices for cloned oil palm that are 2 times the market price for equivalent seedlings. The news media have reported that the Malaysian Government has recently offered 50% rebates for tissue culture laboratory electricity costs in an attempt to encourage increased use of the technology by the industry.

In the conifer timber industry in temperate regions, the plantation managers must wait for a period of up to 30 years before realising a real return on an investment on the higher unit cost of 'superior' planting stock. Despite this the international forestry industry has invested heavily in research and development of forest tree tissue culture technology. Long before somatic embryogenesis of conifers was achieved, companies invested in tree micropropagation (organogenesis). In 1981 Smith showed that the cost of micropropagated *Pinus radiata* was 3-7 times higher than that of seedlings (Smith 1986) and this helped the industry re-evaluate what it could afford to invest in conventional tree breeding in order to attain an equivalent level of cost-effective genetic gain. The forestry industry also invested in automated micropropagation and in somatic embryogenesis research and development in order to find more cost-effective routes to realising genetic gain. By the mid-1990's *Pinus* somatic embryogenesis had become established as an industrial tool for conifer improvement (Smith et al, 1994) and by the end of the decade genetic transformation had been achieved (Walter and Smith, 1999). In the current decade in the Southern USA propagation companies are supplying the forest industry with millions of copies of individual elite genotypes of *Pinus taeda* by a process of somatic embryogenesis (personal communication to DR Smith from industry sources).

In reviews of the role of biotechnology in New Zealand forestry, Smith observed that somatic embryogenesis was more cost-effective than conifer micropropagation (Smith 1997, 1999). This observation is also relevant to the oil palm industry. Although oil palm propagation does not appear to have been subjected to published, rigorous cost-benefit analyses, a general measure of productivity can be readily constructed. Information published in the media and on the Advanced Agriecological Research website indicates that their tissue culture facility employs a workforce of 140 to produce between 1 million and 1.5 million plants per year. This equates to an annual production of between 7,000 and 10,700 plants per worker per annum. The wages of a highly trained work force are a significant component of the total direct costs of any industrial tissue culture laboratory (and note that this excludes the additional indirect costs of housing, medical benefits, education of families and other social costs that many companies have to bear in remote locations.) By comparison, from Table 1 it can be seen that the work-force productivity in forest tree propagation is much higher than the oil palm industry (Smith, 1997, 1999; MetaGenetics, unpublished data).

Table 1: Comparisons of Tissue Culture Laboratory Productivity

Propagation Industry	Plants per Worker per Annum
Oil Palm (AAR, Malaysia)	7,000 – 10,700
Hardwoods (*Eucalyptus, Liriodendron*)	70,000 – 100,000
Pinus sp Somatic Embryogenesis	200,000 – 300,000

In 2006 NBPOL entered into research collaboration with New Zealand interests to assess whether the low-cost *Pinus* somatic embryogenesis propagation protocols could be transferred to the oil palm industry. Initial trials were encouraging and in the last quarter of 2008 a research and development programme was added to the existing tissue culture laboratory at Dami OPRS. We report here progress to date on the development of new somatic embryogenesis protocols.

NBPOL Somatic Embryogenesis Protocols

Palm immature inflorescence explants from only the top field-tested progeny from each breeding family are used to establish embryogenic cultures. The theory behind palm selection is covered in a later section of this report. Inflorescence collection from elite ortets in the field is essentially as described by Vovola and Lord. Immature inflorescences are the preferred tissue type – but this is more a reflection of the smaller amount of damage done to the palm (compared to that caused when removing the crown to sample leaf base meristem) rather than from any inherent superiority of the tissue type used. Access to elite palms in a breeding programme is more likely to receive the support of the breeders if field trials are not disrupted by severe damage to valuable palms.

Immature inflorescences (fronds 8 to 11) enclosed within the spathe are transported to the laboratory on the morning of collection and are immediately surface sterilised and excised as described by Vovola and Lord. Inflorescences are placed into tubes of induction medium as individual spikelets (primary explants). The primary explants are subdivided some weeks later onto proliferation medium (2-3 pieces per spikelet – the secondary explants). Within 12 weeks from palm sampling, direct somatic embryogenesis is observed on the surface of secondary explants. The directly formed somatic embryos (DSE) have distinct root and shoot poles and germinate if detached and placed on an appropriate medium. DSE arise from immature flower meristems, the epidermal surface of sepals, and from inter-nodal epidermal tissue of immature flower spikelets. When left on the secondary explant DSE develop into clusters of compact globular-stage embryos which tend to fuse to form irregular spheres of compact pro-embryogenic tissue (CPEM). CPEM is firm tissue – not at all friable – and breaks into fragments only gradually in liquid culture by budding to form clusters of immature somatic embryos that eventually detach from the 'parent' tissue body. Our observations lead us to the conclusion that this process is secondary direct somatic embryogenesis.

Fifteen different elite *Tenera* ortets were sampled in March 2009 onto a range of induction media and were later subdivided into 2-3 pieces and transferred to various proliferation media. Direct somatic embryogenesis with subsequent proliferation of

pro-embryogenic tissue was observed for all ortets. Most of the pro-embryogenic tissue was observed from only a few of the media tested and so in December 2009 eight different *Tenera* ortets and one *Elaeis guineensis x E. oleifera* hybrid were sampled onto improved media formulations. In this report we focus on the data from the December 2009 *Tenera* sampling.

Pro-embryogenic Tissue Induction Results

Each female Tenera inflorescence yielded an average of 161 individual spikelets on initiation medium (after correction for contamination) and these gave an average of 384 secondary explants after subdivision onto proliferation medium (Table 2). The male inflorescences produced an average of 187 primary explants and 547 secondary explants. The single O x G hybrid produced 93 primary explants, below the range of the Tenera female flowers.

Table 2: Record of Primary and Secondary Explants per Inflorescence from December 2009 Collection

Ortet – Gender	Number of Primary Explants in Initiation Medium	Number of Secondary Explants on Proliferation Medium
T121 Female	200	513
T122 Female	150	302
T123 Female	178	475
T125 Female	116	246
Female Average	**161**	**384**
T118 Male	190	581
T119 Male	200	589
T120 Male	170	452
T124 Male	188	566
Male Average	**187**	**547**
E. guineensis x oleifera Female	93	269

After a total of 12 weeks in culture following sampling, secondary explants were assessed for proliferation of compact pro-embryogenic tissue (CPEM) and an estimate made of the percentage of the surface of each explant covered with CPEM. The mean percentage of each secondary explant forming CPEM ranged from 19.4% to 97.4%. The average surface area of secondary explant covered with CPEM ranged from 0.4% to 44.9% (Table 3). Data was analysed by 2-way analysis of variance using the GenStat (9^{th} edition) statistical analysis package. Treatment differences were very highly significant ($P > 0.0001$). There were also very significant interactions between induction and proliferation media.

For explants derived from female inflorescences, the I4 > P1 protocol was clearly the best, while for male inflorescence explants the I2 > P1 protocol was best. After only 10 weeks on proliferation medium sufficient CPEM had formed that tissue from individual culture tubes could be harvested and combined into shaker culture flasks where the tissue continued to proliferate. This was 17 weeks in total after palm sampling

Table 3: Summary of Compact Pro-Embryogenic Masses (CPEM) on December 2009 Tenera Explants Assessed in April 2010

Gender	Initiation Medium	Proliferation Medium	% Secondary Explants With CPEM	Percent of Secondary Explant Surface Covered with CPEM
F	I1	P1	84.3%	9.3%
F	I2	P1	86.3%	23.8%
F	I3	P1	91.4%	31.4%
F	**I4**	**P1**	**90.5%**	**44.9%**
F	I2	P2	41.4%	1.1%
F	I3	P2	76.2%	2.9%
F	I4	P2	71.6%	4.5%
F	I2	P3	41.4%	1.0%
F	I3	P3	80.4%	3.8%
F	I4	P3	79.0%	3.9%
M	I1	P1	66.7%	3.4%
M	**I2**	**P1**	**97.4%**	**32.5%**
M	I3	P1	84.7%	12.5%
M	I4	P1	92.7%	12.6%
M	I2	P2	26.3%	1.1%
M	I3	P2	40.7%	0.4%
M	I4	P2	53.2%	0.6%
M	I2	P3	19.4%	0.6%
M	I3	P3	38.0%	0.4%
M	I4	P3	49.4%	0.6%

Note that on average each female primary explant (spikelet) on initiation medium was subdivided to form an average of 2.39 secondary explants on proliferation medium. For male explants the subdivision "multiplication factor" was 2.93. Thus it is apparent (Table 2) that there were more tubes with CPEM than there were primary explants. This level of efficiency in rapid induction of pro-embryogenic tissue has not previously been reported for oil palm.

Data from Tables 2 and 3 can be combined to make projections of the potential yield of CPEM assuming that only the most efficient culture medium is used in future. Yield estimates indicate that, on average, a single *Tenera* female inflorescence could

produce 347 culture tubes of CPEM and a male inflorescence 532 tubes (Table 4). This estimate will be recalibrated with future sampling of elite Tenera palms in NBPOL plantations.

Table 4: Estimated Yield Of Elite Tenera CPEM Per Inflorescence Using Best Treatments Only

Inflorescence Type	Total Primary Explants – Mean per Inflorescence	Total Secondary Explants – Mean per Inflorescence	Percentage of Secondary Explants with CPEM	Estimated Tubes of CPEM from each Inflorescence
Female	161	384	90.5%	347
Male	187	547	97.4%	532

Shaker Flask Culture and somatic embryo development - background

Culture of oil palm tissue in shaker flasks has been demonstrated by several groups. In some of the original reports (de Touchet et al, 1991; Teixeira et al, 1993) the processes described conformed to the general definition of authentic somatic embryogenesis. In the following decade however, particularly in Malaysia and Costa Rica, the term somatic embryogenesis began to be used in a less accurate sense (Escobar et al, 2005). Inspection of the illustrations accompanying some later publications referring to 'embryogenic suspensions' indicates that the tissue from liquid culture typically produces shoots and that these are subsequently transferred to medium where adventitious roots are formed later in the process. The classical definition of somatic embryogenesis requires that clearly defined root and shoot poles are formed on the mature somatic embryo. Germination invariably takes the form of the emergence of a root that is indistinguishable from the radicle of a zygotic embryo, followed later by epicotyl or cotyledon emergence. Any deviation from this process is normally referred to as 'abnormal' or 'precocious' germination. By contrast, the product of most recent reports of oil palm suspension culture should be more accurately defined as adventitious shoots arising from nodular meristematic tissue. We concede that adventitious shoots may arise first as attached somatic embryos on the nodular tissue but these are actually difficult to distinguish from immature adventitious shoot meristems during initial development.

Dami OPRS Shaker Flask Culture and somatic embryo development - results

The current Dami OPRS liquid culture project began in June 2009. Drawing from industrial experience with conifer and hardwood suspension culture, proprietary media were adapted to oil palm. *Tenera* tissue from proliferation medium of an elite *Tenera* cell line on hand at the time was placed in 125 ml Erlenmeyer culture flasks and maintained on Ratek orbital shakers. At monthly intervals cultures were screened through stainless steel mesh and the fine fraction plated onto a proprietary embryo development medium. Within 8 weeks dense white immature somatic embryos were seen to form in clumps 3-4 mm wide and with individual embryos never exceeding 1 mm in length no matter how long the tissue was held on this medium.

Individual clumps of these somatic embryos were placed onto germination medium (3 per 20 mm culture tube). Within 2 weeks after transfer root emergence was seen, following immediately by coleoptile emergence. After one week more (total 3 weeks) the individual embryos had grown so much that many had detached from the embryo clusters (became singulated) and much of the tissue mass was lifted clear of the medium by the lowermost embryos. At this point embryos were transferred, one per culture tube, to fresh medium. Five single embryos were produced per millilitre of shaker flask medium in the initial experiment giving us over 600 individual plants. The current yield of plants is around 26% of the immature embryos formed on the embryo development medium. Many more fused embryos were also produced and so yields can be expected to improve in the future as the process is refined.

Although we are not the first to report a genuine somatic embryogenesis process for oil palm, we believe that this is the first report of production of hundreds of oil palm plants where radicle emergence is seen before coleoptile emergence from singulated embryos on a scale that promises to be readily adapted to industry requirements for cloned palms.

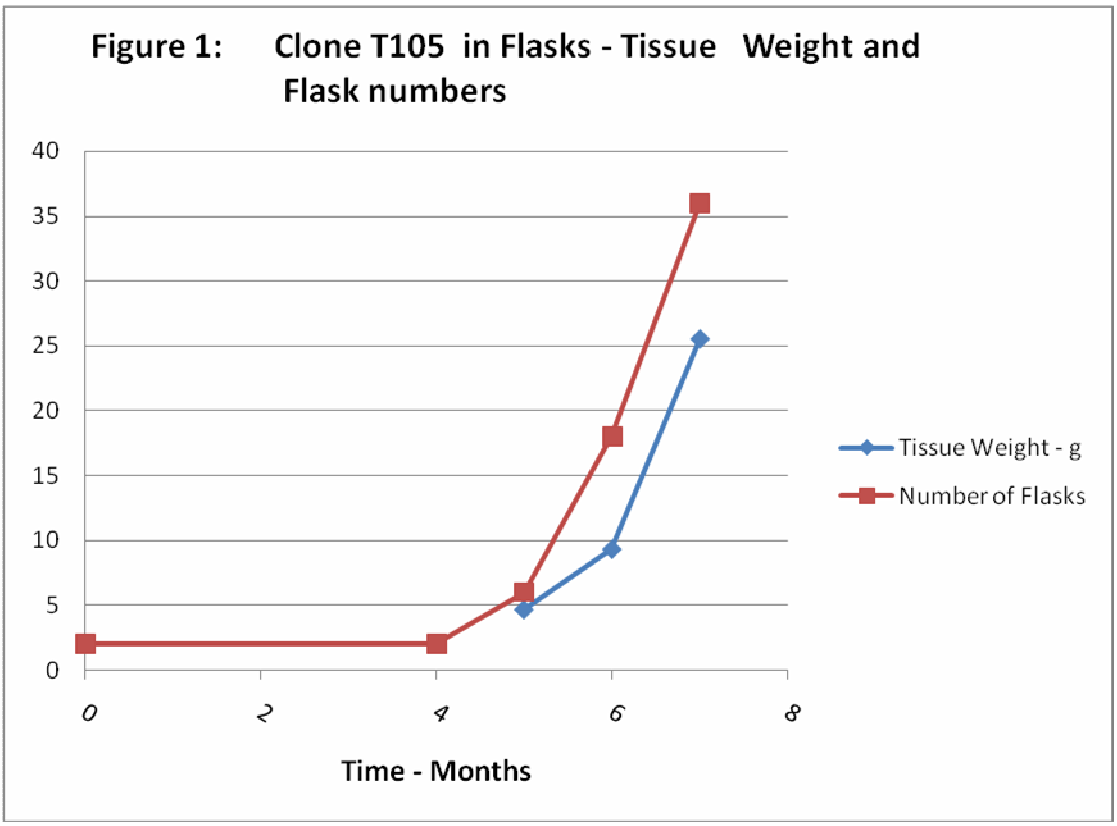
Complete plants from elite *Tenera* have been produced by this process and are now on hardening medium in preparation for transfer to the rametry in the near future. Our laboratory is not currently set up for large-scale propagation from liquid culture because we have not yet made the transition from shaker flasks to bioreactors for the liquid culture step. However we are carrying out shaker flask subculture of embryogenic lines from 12 different elite *Tenera* ortets to see how long we can multiply embryogenic tissue in the shaker flask stage without the development of flower and fruit abnormalities. At present we have a monthly 3-fold multiplication of the tissue if we use flasks for proliferation rather than directly for immature somatic embryo production. With the March 2009 *Tenera* palm lines, an extended lag phase was observed before tissue and flask multiplication began (Figure 1). Our flask inoculation protocols were subsequently improved and the shaker flask lines produced from the December 2009 *Tenera*s seemed to have entered directly into exponential growth with no apparent lag phase.

Multiplication Potential and Deployment of the New Protocols

We now have the capacity to simultaneously inoculate scores of shaker flasks with embryogenic tissue only 17 weeks after sampling a single inflorescence from each elite palm. Flask cultures begin to produce monthly harvest of immature somatic embryos relatively quickly. Based on process efficiencies and embryo to plant conversion rates from preliminary experiments carried out with one elite *Tenera* cell line in 2009, we estimate that a single inflorescence could produce over 22,000 plants from a female flower or more than 30,000 from a male flower that will be ready for the rametry 17 months after sampling. By delaying plant production for two months while allowing multiplication of embryogenic tissue in shaker flasks, we calculate that we now have the potential to produce 1.5 to 1.7 million plants from a single inflorescence ready for the rametry 24 months after sampling.

At the beginning of May 2010 we are testing these projections with shaker flask embryogenic cell lines from all eight of the *Tenera* ortets sampled in December 2009. Samples of each cell line will be harvested at regular intervals and converted to plants

for use in clonal fidelity field trials. It is our intention to eventually explore the use of bioreactors to manage embryogenic cell lines since the large numbers of shaker flasks required for commercial production become unwieldy.



Clonal Oil Palm Deployment

At this point in our research and development programme it would be premature to assume that our product will be free of flower and fruit abnormalities, something that we can ascertain only through rigorous field trials. However we have made progress in both enhancing the percentage of explants that form embryogenic tissue and in reducing the time that it takes to begin transfer of substantial numbers of hardened plants to the rametry. Given these improvements it is appropriate to begin planning how to deploy clonal material in our plantations.

Vegetative Amplification

Soh and co-workers (2001) rightly point to the dangers of assuming that the apparent high yield of fruit on a single phenotypically superior palm will always be expressed when that palm is cloned and deployed in a plantation. The greatest risk lies in selecting only one or a few palms and multiplying them extensively without first testing their field performance as clones. Forestry in New Zealand faced the same situation in the 1970's when micropropagation was first developed for *Pinus radiata*. The solution was the development of a new concept – "vegetative amplification" (Smith et al, 1981). A small amount of rare and expensive seed from progeny-tested

controlled crosses (controlled pollination) was used to micropropagate a number of clones. This untested juvenile material was planted knowing that the yield gains from the superior seedling family would be delivered over a larger area of the forest estate, using the cloned plants, than was possible using the limited amount of seed available. The average genetic gain of the family could be realised even when some individual clones performed poorly provided that enough genotypes were used. In recent years the concept of vegetative amplification is now carried out using juvenile cutting stool beds (often from cryo-preserved somatic embryos) and has been widely adopted in New Zealand and elsewhere where it is now known as "family forestry".

Soh et al (2001) described how the concept of 'vegetative amplification' of untested seedling oil palm has been tested with oil palm and observe that the genetic gain will be no higher than the family mean. We agree with their observation however we note that since NBPOL advanced breeding families produce the equivalent of 3-4 tonnes CPO per hectare more than our standard plantation seedling stock, capturing this improved gain over a substantial part of our replanted estate offers significant future economic gain to the company.

We believe that a modified form of vegetative amplification could be used to spread genetic gain over a wider area of plantation than is possible when the number of elite parents in a seed garden is the main limiting factor. The best progeny in a breeding trial can be selected based on the quality and form of their fruit bunches and fruit and oil yield after 5-8 years of evaluation. After 'mass propagation' through tissue culture the plants can be used in plantations without maintaining their clonal identity. In general the mean performance of such a 'mass propagated mosaic' would probably exceed that of the family from which they were selected since the ortets could all be selected for their position well to the right of the normal distribution curve of the elite field-tested seedling population from which they were selected.

As an example of the potential we look to the 8 ortets that we used in our December 2009 palm sampling. Each palm came from different progeny trials for it is our policy to keep a wide genetic base in future plantations. The mean progeny-tested CPO yield per hectare of the families that we used was calculated to be 8.65 tonnes /hectare /year (range 7.70 – 9.85). On the other hand, the mean estimate for CPO from the chosen ortets based on their individual bunch analysis data collected over 8 years was 11.45 tonne/hectare/year (range 10.08 – 13.71). When this sampling pattern is repeated several times but with different ortets from different breeding trials, the average CPO production of the selected ortets will tend towards substantially exceeding the mean of the families that they were selected from.

Companies that wish to obtain additional genetic gain could establish clonal field trials at the same time with the viewpoint of re-cloning from verified superior genotypes in future years. At present there is just not enough reliable data published in the literature to allow us to carry out a cost-benefit analysis to compare genetic gain from using a declining number of 'superior' clones (the re-cloning technique) contrasted with the option of 'mass vegetative amplification' from an advancing series of palm breeding trials. Certainly the 'vegetative amplification' route will spread any risks from flower abnormalities, unusual disease susceptibility, or annual periods without fruit production – all features seen in at least some oil palm clones over the years. Furthermore, without the 'compliance' costs of tracking and recording

individual clone performance, management costs of 'vegetative amplification' will be lower than for clones.

Conclusion

The recent developments in palm cloning efficiency at Dami OPRS give to NBPOL new opportunities in realising real CPO production gains in our plantations in the near future. The relative merits of 'vegetative amplification' of elite progeny compared to the use of individual elite clones will become clearer as our research and development programme proceeds.

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