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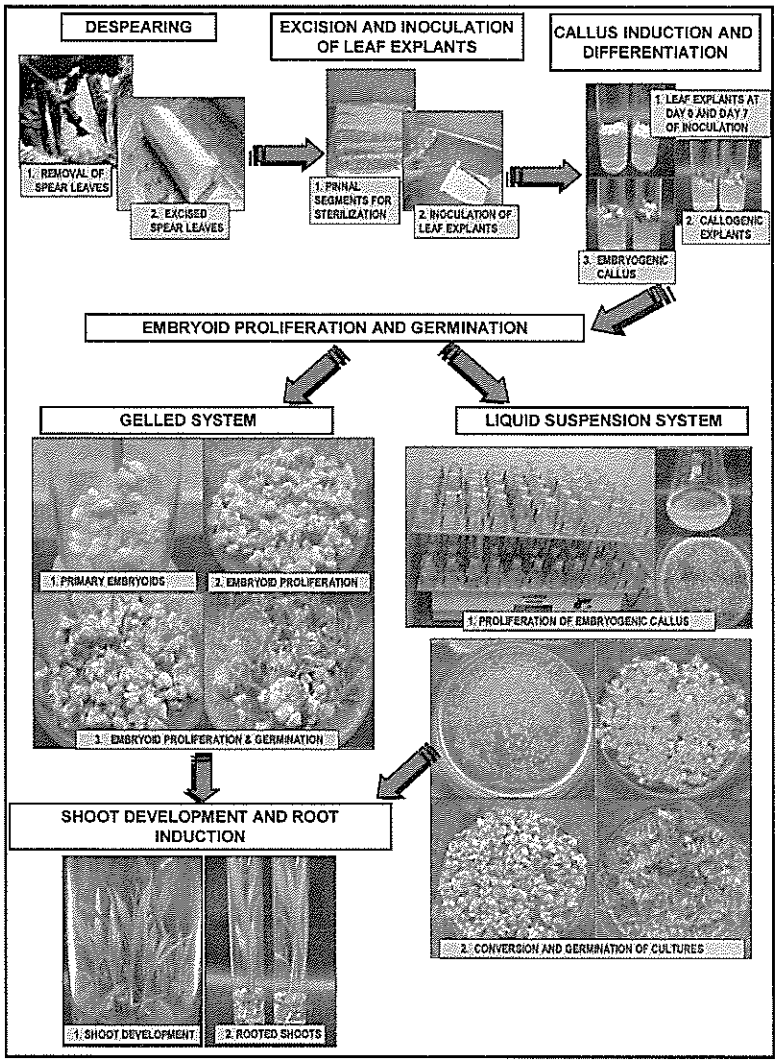

Figure 5. Oil palm tissue culture process: gel and liquid suspension culture systems

having no natural means of vegetative propagation. But it has taken three decades of R&D to become a technology due to a number of critical issues that needed resolution [19]:

1. Inefficient cloning technique (Figs. 4,5). Only 18% of the selected palms cultured can give rise to mass propagating cultures. The gel culture is inefficient in terms of slower and asynchronous culture growth and development and thus not so amenable to automation. The liquid suspension system is needed for large scale propagation [19].
2. Risk of somaclonal variation (Fig. 6). The risk of somaclonal variation in the form mantled parthenocarpic fruits resulting in bunch abortion and sterility has been the main stumbling block for the commercial production of oil palm clones for the past two decades [20]. Susceptibility varies between and within clones and the risk tends to increase with extended culture, recloning and liquid culture [19,21,22]. Through empirical research in protocol refinement, culture selection and strict process control in the laboratory and producing a package of clones each time, AAR has succeeded in the mass propagation of proven clones particularly by the liquid culture system with minimal risk (<3%) of abnormal palms [19]. Reflecting this confidence, a

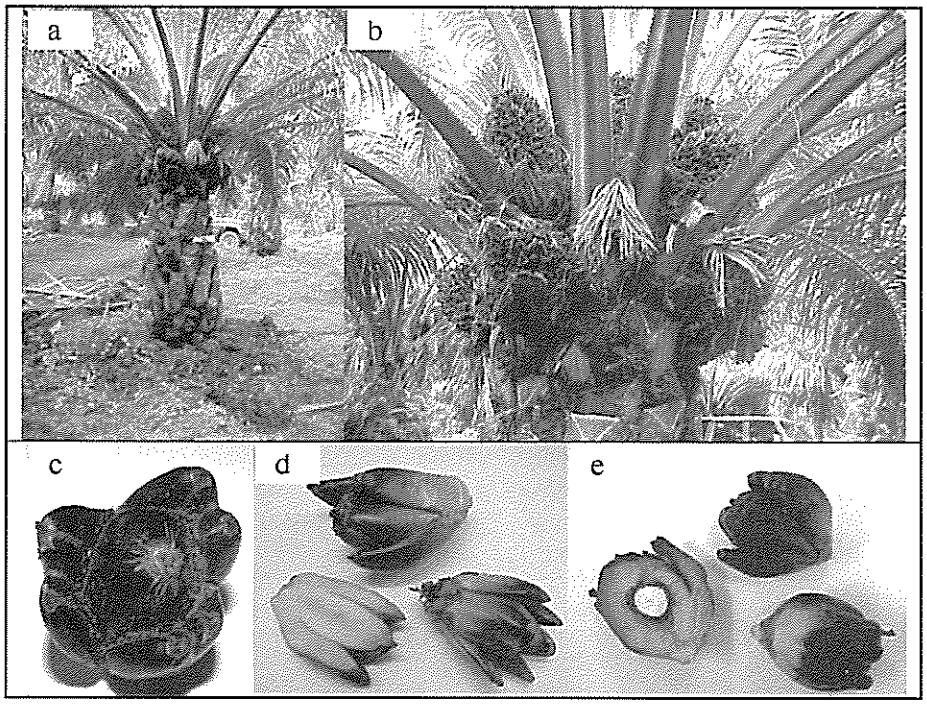

Figure 6. Mantled fruit somaclonal variant in oil palm. (a) Sterile palm with abortive mantled fruit bunches (b) Palm with unaborted mantled fruit bunches (c) mantled fruit with the vestigial stamens in the female flowers developed into fleshy carpels to form a mantle surrounding the fruit (d) parthenocarpic mantled fruits (e) fertile mantled fruits.

new commercial laboratory capable of one million plantlet production annually has been commissioned.

3. Low frequency of outstanding clones. AAR has planted more then 200 ha trial and 9000 ha commercial clone plantings. In the commercial plantings (Tables 4-6) the advantage of clones over the dura x pisifera or DxP hybrids in fresh fruit bunch (FFB) yield (ca. 23%), oil extraction rate or OER (ca. 15%) and oil yield or OY (ca. 41%) was demonstrated. To separate crop age bias in OER, separate mill runs were made (Table 7) which demonstrated that young clone plantings gave higher OER than young DxP plantings and the latter better OER than the older plantings reflecting the genetic progress made. Commercial FFB yield figures tended to bias in favour of clones as early clone plantings were planted on better areas and given additional inputs presuming their higher yield potential. Trial results

Table 4. Comparison of fresh fruit bunch yields (t/ha) of oil palm clone vs DxP hybrid plantings in Segaria Estate.

Material	Ha	2004	2005
(1) 1998 Planting			
AA clones	212.9	25.9 (132%)	27.8 (134%)
AA DxP	30.6	19.7 (100%)	20.7 (100%)
(2) 1999 Planting			
AA Clones	240.2	20.6 (99.5%)	25.4 (113%)
AA DxP	96.5	20.7 (100%)	22.5 (100%)

Table 5. Comparison of fresh fruit bunch (FFB) yields (t/ha) (1999 plantings, 2005 yields) of oil palm clone vs DxP hybrid plantings in KDC Estates.

Planting Material	Ha	FFB Yield	Highest Yield	Lowest Yield
AA clones	249	33.7 (123%)	39.8 (115%)	31.3 (157%)
AA DxP	1,622	27.3 (100%)	34.6 (100%)	20.0 (100%)

Table 6. Comparisons of mill oil extraction rates (OER) of oil palm clone vs DxP hybrid plantings in Segaria and KDC estates.

Mill	Crop	Ha	Total FFB* (t) Processed	OER (%)
SEGARIA Mill	Clone	464.3	390.0	24.3
KDC Mill	DxP			22.4
	Clone	423.0	343.0	26.7
	DxP			21.9

* Fresh fruit bunches

Table 7. Comparisons between mill oil extraction rates (OER) of oil palm clone vs young DxP hybrid vs old DxP hybrid plantings.

Item	AA Clone 1999 Planting	AA DxP 1999 Planting	Mixed DxP 1978-79 Planting
FFB processed (t)	388.7	339.6	502.9
OER (%)	25.6	22.1	19.7

FFB – Fresh fruit bunches

Table 8. Summary of recent trials comparing fresh fruit (FFB) and oil yields (OY) of oil palm clones vs commercial DxP hybrids as controls.

Trial	Year of Yield data	Clone FFB Yield		DxP FFB yield (t/ha)	No. of Clones > 10% > DxP	Clone OY		DxP OY (t/ha)	No. of Clones > 10% > DxP
		Range (t/ha)	Mean (t/ha)			Range (t/ha)	Mean (t/ha)		
BCT 13-97	2000-2005	21.7-27.2	25.8	20.9	8/11	5.7-7.6	6.7	5.2	11/11
BCT 14-97	2001-2005	20.7-29.2	25.7	23.9	5/9	6.3-8.9	7.6	6.5	7/9
BCT 15-98	2000-2005	21.0-28.5	28.5	25.7	1/7	6.2-8.9	7.4	6.3	6/7
BCT 16-98	2001-2005	13.9-23.7	19.4	17.1	14/23	4.1-7.2	5.8	4.7	18/23
BCT 18-00	2002-2005	20.3-31.8	25.2	25.2	10/18	5.9-8.6	7.4	6.8	14/18
	Mean		24.4 (109%)	24.2 (100%)			7.0 (118%)	5.9 (100%)	

would be more objective. In five recent trials (Table 8), on average the clones were better than the hybrids by 9% for FFB yield as compared to18% for OY. There were many clones lower FFB yielding than the hybrids as compared to OY. The frequency of outstanding clones was also lower because of inefficient ortet (source palm) selection (particularly for FFB yield) due to the masking of the genetic differences by environmental effects i.e. low heritability of yield [23]. Hence outstanding clones can only be identified after clone testing. By which time the original cultures are no longer available and the original palm is not so amenable for cloning. Recloning of the proven high yielding clones is thus needed.

4. Clonal hybrid seeds. Owing to the earlier uncertainty in the clonal propagation of elite palms by tissue culture, an alternative strategy adopted by some laboratories is to clone the parents of superior hybrids for mass clonal seed production [19,24]. As only limited clonal parents need to be regenerated and that commercial seeds are produced by the normal sexual process which could screen out deleterious mutations, somaclonal variation risk would be minimal. Commercial clonal seeds are now available.
5. Dihaploid parents. If haploids could be found or induced from the parents of superior hybrids, dihaploid fully homozygous parents, similar to fully inbred parents sans the protracted inbreeding process (five generations) for commercial F1 hybrid production, could be obtained e.g. by colchicine treatment. Research efforts to obtain haploids are in progress (Maria, Maddon per. comm.).

FURTHER ISSUES

The oil palm industry is about to enter a new era of large-scale plantings of more genetically uniform materials in the form of clonal hybrids and clones. Nevertheless, there are still issues that need to be addressed and investigated to ensure successful exploitation of these technological developments and minimize risk of catastrophic setbacks.

In vitro propagation

It would be useful to demonstrate the heritability of clonability and susceptibility/resistance to abnormality and their repeatability in clones and reclones and to identify the predisposing environmental (in vitro/ex vitro) factors. Solving these issues, perhaps with the development and assistance of molecular markers, would facilitate the selection and breeding of amenable and resistant genotypes for cloning. Safety limits for mantling risk and reduced fitness for cycles of recloning and for levels of production especially for the liquid culture technique [25] should also be established with the help of molecular markers. Prospects for optimization and automation of the tissue culture protocol particularly the liquid system are still good and necessary if clones are to make significant inroads into the large annual national (ca. 50 million) and international (ca. 200 million) oil palm seeds market. The current R&D to fully develop the bioreactor system and synthetic seeds are appropriate [26]. There is also need to demonstrate that clonal hybrids do not perform poorer than sexual hybrids because of reduction in fitness of the clonal parents [27,28].

Field testing

Clonal hybrids and clones being genetically more homogeneous and discrete than mixed hybrids, would respond differently to different environmental factors, e.g. location, planting density, fertilizer requirement, and abiotic (soil, water, mineral) and biotic (pest, disease) stresses i.e. exhibit genotype x environment (GxE) effects (29-31]. Adaptability trials are thus mandatory.

For clonal hybrids, the choice of the hybrids should be based on GxE tests of the original sexual parental hybrids. In the case of clones which are more prone to GxE effects and where ortet selection is inefficient, repeat GxE tests may be necessary to pick out the superior clones. To reduce the time and effort in GxE testings for a perennial tree crop, the selection of a minimum sample of environments that can discriminate the differential responses of the genotypes yet representative of the major environments where the genotypes are

to be grown warrants investigation. For continuous variety improvement, production or reproduction of superior families, preferably of different genetic origins and in larger numbers, is needed to provide the ortets for cloning. Large scale plantings of genetically homogeneous materials of restricted genotypes would also lead to genetic vulnerability of the crop to epiphytotics, pest outbreaks, inadequate pollination and moisture and mineral deficiencies. Thus, research is required for the packaging of clones and their field planting arrangements to reduce genetic vulnerability and to also synchronize the cropping pattern with prospective mechanized harvesting, and new crop management systems.

Transgenics

Putative transgenic high oleic, high stearic, high ricioleic, high palmitoleic, and high PHB/PHBV oil palm cultures and plants have been obtained and are contained in a biosafety greenhouse awaiting regulatory approval for field testing [32]. Being mediated by biolistics, most of the transformants would not be useful having variable copies of the transgene. *Agrobacterium tumefaciens* mediated transformation, the preferred approach, is being researched. Also since the transformed palms have been regenerated from tissue culture, the transgene may be silenced [33]. These underscore the importance of field tests at least in the initial technique development stages to prove the reliability and efficiency of the process.

Marker assisted selection (MAS)

Molecular markers would be very useful in oil palm breeding, seed production and tissue culture: DNA finger-printing for parent authentication for efficient breeding and for plant variety protection of hybrids and clones; quality control in seed and clone production e.g. genetic contamination from illegitimate pollination in seeds, somaclonal variation in clones and seed and culture mix-ups; early and efficient selection for desirable traits at the laboratory and the nursery stages [34,35]. The presence of D palms with thick-shelled fruit is evidence of contamination by illegitimate pollination in DxP (or tenera) hybrid progenies. Markers for the shell gene have been mapped but were still not close enough to prevent recombination for a reliable selection marker but research efforts continue [36,37]. A reliable marker for the virescens trait (fruit green when unripe and orange when ripe) which facilitates identification of a ripe bunch has been obtained. Efforts are ongoing in identifying/ verifying markers/quantitative trait loci (QTL) for oil quality traits (oleic acid, stearic acid, myristic acid, palmitic, palmitoleic acid), and embryogenesis traits in oil palm tissue culture.

The mantling abnormality persists as an element of risk in large scale clonal palm production requiring resolution. Much research has done on its physiological, genetic or epigenetic (gene expression) basis [38-42]. The current explanation based on molecular research at MPOB and its contracted international partners favour an epigenetic phenomenon involving methylation probably of the homeotic (flowering) MADS box genes [43-46]. A molecular marker procedure for early screening is the final objective. To date a number of putative markers identified turned out to be clone specific or dependent on host genotype. A number of loci or genes may also be involved and much research remains.

A number of putative quantitative trait loci (QTL) for callogenesis and embryogenesis has also been found to map close together (Rajinder Singh, pers. comm.). If confirmed, this would open up the feasibility of MAS of palms and tissues amenable to tissue culture clonal propagation besides tolerance to mantling. Meanwhile, AAR is identifying clones which are stable or resistant to somaclonal variation which could subsequently provide genetic materials for the development and validation of molecular markers or genes for this trait.

Development of MAS/QTL for yield selection is being attempted at MPOB and would prove to be a daunting task as in other crops [47-48] as yield is a quantitative trait with low heritability and is susceptible to GxE effects. MAS would be useful in yield selection indirectly in backcross breeding programmes by selecting for the recurrent high yielding parent host genotype in segregant plants with the donor trait incorporated and in organizing breeding parents into heterotic groups in hybrid breeding [49-51].

It is evident that while improved cultivars is the surest way to improve the productivity and sustainability of oil palm production, it will still take time despite the current emphasis on biotechnology,

as the tools need time to develop. Even if the tools and cultivars are available now, their full impact can only be seen in the field some 20 years later as plantations cannot afford to replant more than 5% of their fields per year. In other words, there is still a large proportion of old plantings whose yield or productivity needs to be urgently improved through non-genetic means i.e. improved agro-management practices!

AGRONOMIC IMPROVEMENT APPROACH

Further improvement in palm productivity can come from either increasing yields through R&D and adoption of new production technologies or sustaining the current yields but with reduced inputs through increased production input efficiency.

Fertilizer nutrients and productivity

Marked improvements in oil palm yields had been achieved over the past 4-5 decades through breeding, fertilizer application, advances in agronomic practices as well as improved milling efficiencies [52]. The largest yield improvement (93%) has come from increased and balanced fertilizer application. Yield data from a number of trials indicated that when nutrients are non-limiting comparable high yields could be achieved on a wide range of soils [53]. Adequate nutrient input is the key to sustain high yields.

Advances in fertilizer management

Two important developments in agronomic research have facilitated the shift to site-specificity and precision agriculture i.e. (i) the concept of site yield potential [54,55] and (ii) the concept of nutrient balance [56] for estimation of fertilizer requirement.

Application of the concept of site yield potential (SYP) has facilitated setting of realistic yield targets and a multidisciplinary approach to yield maximization. It helps focus attention on identification of yield constraints and site-specific inputs to alleviate them. This allows more accurate and site specific fertilizer rates to be formulated to meet target yields and avoid excessive and under-application of fertilizers. The simple nutrient balance concept provides a logical, quantitative basis for estimating fertilizer nutrient requirements taking into account the multi-factorial components of nutrient demand and supply in the oil palm system. The nutrient balance approach therefore provides a framework for agronomists to integrate the many factors that need to be considered when formulating fertilizer recommendations. It also complements the SYP concept in the transition to site-specific fertilizer management.

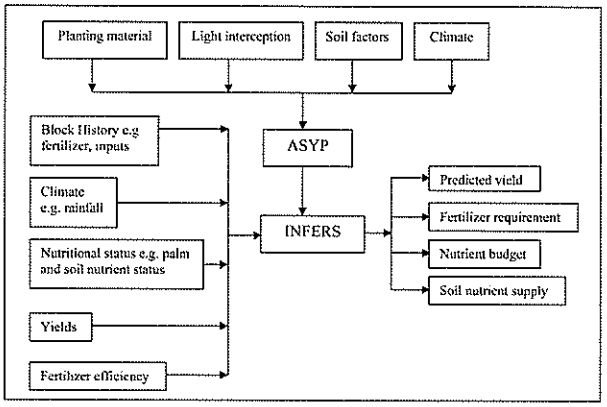

Figure 7. Schematic layout showing input and output parameters for INFERS. Source[56].

A logical progression from the nutrient balance approach is the development of an integrated site-specific fertilizer recommendation system (INFERS, Fig.7) for efficient fertilizer management in oil palms [56]. INFERS incorporates ASYP (AAR's SYP) model as an input parameter and also integrates all relevant data such as soil and leaf analysis, vegetative growth measurements, rainfall, previous fertilizers applied, yield records, as well as other site specific data such as soil type, texture, terrain, drainage, etc. The ASYP module sets the target yield to be achieved and the other input data compute the estimated nutrient demand and supply needed to achieve the projected SYP.

Further advances led to the development of a holistic Integrated Agronomic Management (IAM) system for oil palm nutrient management [57]. In this system, INFERS is linked to a comprehensive relational database (AA AeGIS™) which stores all the information necessary to compute the nutrient requirements of palms for any given site, taking into account site factors such as soils, terrain, rain fall, yields and past fertilizer history amongst others. Global positioning system/geographical information system (GPS/GIS) modules allow sites to be characterized more precisely. The

requirements for balanced nutrient inputs can be derived accurately, rapidly and objectively for optimal growth and yield. A 'best months, timing and allocation' module determines the best month for application and allocates rates and application schedule to minimize potential losses. Finally a comprehensive report is generated with appropriate site-specific recommendations. With this integrated agronomic management system, fertilizer losses are minimized and efficiency is maximized through correct and timely applications.

Good agricultural practices and sustainability

Good agricultural practices (GAP) which improve the efficiency of fertilizer uptake and use will also enhance crop sustainability. These include practices that minimize potential losses, preservation of soil quality, effective soil and water conservation, waste management and recycling of palm residues and palm oil mill by-products, zero-burn land preparation, ground cover management, water management and integrated pest management (IPM).

Soil and water conservation practices. Planting of a fast creeping legume cover crop as soon as possible after land clearing (when soil and nutrient losses are probably greatest) is generally a standard practice. The recent introduction of *Mucuna bracteata*, a shade-tolerant legume cover crop, is expected to also improve fertilizer efficiency by reducing palm dependence on inorganic N fertilizers.

Construction of planting and conservation terraces is a basic necessity when planting on sloping land with slopes of >12° and >6° respectively. Terraces reduce the slope length and runoff velocity and consequently erosion and nutrient loss. Stop bunds at regular intervals along terraces will reduce lateral flow and runoff.

Pruned fronds and empty fruit bunches (EFB) provide excellent organic mulch for very effective soil conservation. Stacking of pruned fronds across the slope can reduce soil loss by 30-50% and up to 77% on steeper terrain [58,59].

Ground cover management. Another critical period of high risk of fertilizer losses is when ground vegetation is sparse due to dense shading from the palm canopy or excessive chemical weeding. Thinning out very tall, etiolated, unproductive palms improve yields and encourage ground cover regeneration. Except for palm circles and paths, maintenance of a reasonable ground cover of soft grasses and ferns throughout the productive life of the palms to minimize surface erosion is a prudent practice.

Nutrient recycling. Recycling of the nutrients in palm residues and by-products e.g. palm fronds, palm oil mill effluent (POME) confers numerous benefits and advantages and contributes greatly to the sustainability of the crop. They substitute for inorganic fertilizer and also return large quantities of organic matter to the soil. Very significant quantities of nutrients are already being recycled annually in the plantation through pruned fronds [60]. Nutrient release from pruned fronds is rapid and within 24 weeks, as much as 14% and 24% of the annual N and K requirements of a high yielding mature oil palm field is recycled. The organic materials also replenish the soil organic matter and fertility. Indirectly, recycling also reduces fossil fuel consumption (in producing fertilizers) and pollution (from incineration of EFB).

Zero-waste, zero-discharge technology. A novel patent-pending composting technology developed by AAR and Boustead recently, is able to convert all the POME produced into an organic fertilizer by composting and bio-drying using EFB as the bulking material [61]. This new technology dispenses with the conventional anaerobic effluent ponds and the associated methane emission. This truly 'zero-waste zero-discharge' technology is expected to be widely adopted and will further improve the recycling of nutrients, help to protect the watercourse and environment, and enhance the sustainability of oil palm plantations. Through the Clean Development Mechanism (CDM), there is also the possibility of added revenue from sale of certified emission reduction (CERs) for the industry.

Zero-burn land preparation. Zero-burning replanting is now mandatory in Malaysia with legislation prohibiting open burning. During replanting, palms are felled and shredded or pulverized. With zero-burn replanting, this large pool of nutrients is therefore returned to the field [62]. However, this sudden flush of nutrients particularly K from the

decomposing biomass will not be fully taken up by the next generation of young palms. One way of overcoming this is to reduce/withdraw fertilizer (e.g. N and K) applications up to three years before palm felling [63]. An alternative strategy is to establish a dense legume cover crop as soon as possible after land clearing to immobilize the nutrients released However, due to its sheer size a large proportion of the nutrients will remain untapped and effective ways to conserve this must be developed urgently.

Oil palm plantations and biodiversity

The palm oil industry has developed and implemented GAP that are environmentally-sound and sustainable. Despite this, the palm oil industry has been linked in recent years to the destruction of tropical rain forests and loss of biodiversity. Henson and Chang [64] made an objective appraisal of the link between recent forest loss in the tropics and expansion of oil palm plantings in the tropics. They concluded that the causes of forest loss were multiple and complex and that world wide, the expansion of oil palm plantations during the 1990s accounted for less than 3% of the forest area lost in the tropics. Similar conclusions were reached by others [1,65]. Notwithstanding this, most of the forests converted into oil palm were already logged over and much degraded.

Although oil palm cultivation in general poses little direct environmental threat *per se*, there was undoubtedly a significantly lower number of plant and animal species in an oil palm plantation compared to natural forests [66]. An attempt was made recently to compile an inventory of the flora and fauna in an established coastal oil palm plantation in Selangor [67]. Results showed a surprisingly high number of flora and fauna species recorded in one year: 86 species of flowering plants (43 families) in Site 1 and 80 plant species (49 families), 20 bird species and 7 fish species in and around the swamp in Site 2. These studies indicated that even relatively small areas of undisturbed habitats in the plantation ecosystem can harbour a significant array and variety of plant and animal life including rare and protected species as well as refuge for migratory birds. Although biodiversity in such relatively small conservatories still cannot match those of undisturbed rainforest ecosystems, they are nevertheless important and viable means of conserving the immensely rich biodiversity of the increasingly threatened lowland tropical forest ecosystem.

Some practical ways that estates can conserve and enhance the biodiversity of the plantation ecosystem are [68]:

(a) Conservation of the natural vegetation within the riparian zones along rivers and streams;
(b) Setting aside permanent green belts at strategic locations e.g. steep areas of >25° slopes, rocky hills and other areas unsuitable for oil palm planting; and
(c) Maintenance of water catchments and conservation of natural wetlands, swamps and other water bodies within the estate.

These relatively simple measures will not only enrich the biodiversity of the plantation but can even be advantageous by excluding these 'problem areas' from the productive area of the estate. These options together with the many existing good agricultural practices (e.g. planting of beneficial plants for IPM, use of biological control agents for pests and diseases, no burn replanting etc) can contribute significantly to the conservation and enhancement of biodiversity of tropical lowland forest ecosystem.

Future agronomic needs and approaches

The future emphasis and direction must be the continuous adoption and application of new technologies and the use of the latest scientific results to refine the current best practices to ensure that they remain efficient and sustainable. Continued R&D is an important driver towards these goals and must therefore be given the necessary support and priority. Some specific needs are:

1. Further development and refinement of site-specific and precise approach to nutrient management for long term soil fertility and efficient use of mineral fertilizers. The objective is the maintenance of soil fertility and plant nutrient supply at an optimum level for sustaining the desired crop productivity through optimization of the benefits from all possible sources of plant nutrients in an integrated manner.
2. Increase recycling of palm biomass residues and mill by-products and wastes to improve efficiency of nutrient recycling and

utilization including research on biological and chemical processes for optimizing nutrient cycling and recovery.

3. Greater exploitation of biological processes e.g. N-fixation by legume covers, direct inoculation of N-fixation organisms, mycorrhizal associations, etc, to reduce dependence on inorganic fertilizers and to improve nutrient uptake and utilization.
4. Improved techniques to assess soil nutrient supplies.
5. Selection and development of nutrient efficient, high-yielding planting materials including clones.
6. Adoption and development of integrated agronomic management systems to expedite decision-making and facilitate implementation of site-specific and precision nutrient management practices.
7. Development and use of appropriate indicators of soil quality and nutrient use efficiency to enable monitoring and evaluation of progress and sustainability.

Precision agriculture

The rapid development and achievements in computer science, information technology and mechatronic engineering have spawned a new prospect to fine-tune the management of oil palm and decision making in the plantations. The combined use of these technologies called precision agriculture (PA) is supposed to enhance the efficiency of utilizing natural resources in the plantations or external inputs e.g. fertilizers, at a fine scale in order to maximize profitability per land area. Although the tools and technologies associated with precision farming have been widely utilized, it has not been implemented in oil palm plantations per se. This is because PA is a cropping system which cannot be separated from the management system and philosophy of the plantation i.e. creating yield map alone does not constitute PA. It also involves large capital expenditure, re-training of personnel including workers, and human perception and acceptance of new practices, and has yet to be proven economical in oil palm plantations. Despite the above, the aim of PA and its potential benefits to the plantations such as maximizing FFB yields, optimizing inputs through precise actions, identifying areas for replanting and planting, and objective monitoring and assessment of results, warrant a concerted research and development effort by the industry to prove its values or otherwise.

The central tenet of PA is identifying variability and managing it through precise actions [69]. In oil palm plantations, the main variabilities of interest are FFB yields and soils. The former is commonly the sole economic produce from the plantations and the single most important factor influencing profit whereas the latter is the medium for palm growth and production and it affects fertiliser input, the largest cost item. Goh *et al.* [70] using geostatistics showed that the coefficient of FFB yield variation of individual palms in a fertiliser response trial of about 25 ha can be large at about 35% while those related to soil nutrients such as N can exceed 45%. The random (nugget) variation usually accounted for less than 26% of the total variation which implies that PA is applicable to oil palm.

Before PA can be adopted by the oil palm industry, we need to understand and quantify spatiotemporal variation, which is a natural feature in perennial crops, and seek solutions to manage it. For example, we can reduce spatiotemporal variation of FFB yield by reducing the size of management zone [69] although the optimal size is still debatable. Related to this, a detailed study of spatial reasoning which is a method for human and computer to make inferences about spatial aspects of the environment would be useful. This is because the computational problems associated with PA increase exponentially with finer spatial scale due to the shear amount of information to be processed and the compounding errors from the inherent error of each data involved in the computation. This is an active area of research in the West as the challenges involve not only an understanding of the crops but necessitate the development of new mathematical or statistical principles, and computing theory e.g. hotspotting and fuzzy neural network.

PA will become feasible only if most of the plantation operations are mechanized. Of utmost importance is solving the cutting mechanism and reaching the tall palm mechanically, and the development of an all terrain vehicle to maneuver soft ground e.g. peat swamp and steep terrain. With machine, data-loggers, yield monitors, GPS, sensors etc can be fitted for data collection. However, the

errors and precision of the data and generated yield maps need further investigation. The current high cost of diesel also demands a closer scrutiny of mechanization in terms of cost:benefit ratio and machine efficiency.

There is much scope for management improvement through PA particularly in increasing labour productivity via better infrastructure, more precise planning, organization, budgeting and worker friendly practices [68]. Ultimately, it is the management who will make PA in oil palm plantations a success or failure.

Modelling

Advances in computer technologies have led to rapid development and application of quantitative and mechanistic models that allow evaluation of complex physical, chemical and biological processes [70]. The main aims of this abstract representation of reality are to understand the system behaviour and identify key processes or attributes which can be manipulated for one's own purpose. A good example is the use of model by the International Rice Research Institute (IRRI) to identify the major characteristics and architecture of Leaf 3 in paddy which will support large panicles and supply sufficient carbohydrates to fill the grains fully. This has led to the development of Super Rice [7]. Similar work should be done for oil palm in order to raise its genetic yield potential and consequently its SYP and actual yield. While light model has been developed for oil palm, there is no mechanistic model for water and nutrients, which are major growth limiting factors of oil palm. The latter are also the largest cost item in the production of oil palm. These models could probably assist in the understanding of the processes and suggest appropriate measures to optimize their usage efficiency in the plantations.

Graphic programming tools such as Visual Toolkit have enabled the creation of realistic 3D models of plants in particular their architecture as they grow. They also provide insights into how a plant reacts to various constraints and environments. Such models have also been developed for oil palm but unfortunately, at present none has been integrated with known functional processes to allow better system analysis of the biophysical aspects of the palms other than 3D visualization.

Decision support system

Decision support system (DSS) translates data into knowledge for differential actions in the fields generally by combining database, crop models, expert system and artificial intelligence. The primary aim of DSS is not to make decision for the management but rather to provide a set of likely solutions or options for the management to choose from in order to solve his problems. Thus, it is one of the many tools for people to make better decisions. A number of DSS has been developed for oil palm plantations [71] to assist decision making and implementation of site-specific fertilizer recommendations and agronomic practices

With the current computing power including faster processing speed and larger memory, development is now towards spatiotemporal information system where changes in time and spatial dimension of each attribute in the database are tracked. The difficulty here is in handling uncertainty and error associated with each data and their combined effects on the accuracy of the final output. The next phase of information system is likely to be web-based and mobile-based using robust Pocket PC that are armed with in-built GPS, digital camera, telecommunication facility and wireless with 3G or better technology.

CONCLUDING REMARKS

The per capita intake of edible oil is still low in many developing countries. The emerging economies of some of these large countries e.g. China, India are accelerating and as such, the demand for edible oils will escalate likewise. With increasing affluence attained in these countries, the demand for energy particularly for vehicular use will also escalate. There is currently a large and increasing interest in the development of biofuels, initially responding to meeting the requirements of the Cartagena Protocol in reduction in carbon gas emissions by the First World countries but subsequently because of the imminent depletion of fossil oils and their rising prices. Palm oil whether in its crude form or as methyl ester or biodiesel, can be blended with petroleum diesel in various proportions to run diesel engines. The market size for diesel is 700 million tonnes and the only economically feasible, renewable and expandable source of oil which can significantly

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(although still low) meet some of the requirements is palm oil. Furthermore, with committed and continuing efforts in R&D and innovation presently practised and in the future, palm oil will be found to be more and more sustainable with more and more uses found for its oil and byproducts which are green and renewable, to have even better GAP, to help conserve biodiversity and consequently improve the lives of the rural communities and the country's economy. Undoubtedly, the palm oil industry will continue to remain sustainable if not more so.

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Ultrastrutural studies of chromatophores of the Siamese Fighting Fish (*Betta splendens*)

Gideon Khoo[1], **Lim Tit Meng**[2] and **Violet P. E. Phang**[2]
[1]Faculty of Engineering and Science, Universiti Tunku Abdul Rahman,
Setapak, 53300 Kuala Lumpur, Malaysia
[2]Department of Biological Sciences, National University of Singapore,
Kent Ridge, Singapore 117543
(Email: gideonkhoo@mail.utar.edu.my)

Abstract The ultrastructural morphology of the chromatophores of the Siamese fighting fish (*Betta splendens*) was examined by transmission electron microscopy. Erythrophores, xanthophores, melanophores and iridophores were observed in the epidermis and dermis. Specific combinations of these chromatophores formed the pigmentation patterns of different strains. The ultrastructure of erythrophores was characterized by ellipsoidal electron-lucent vesicles that had a limiting membrane and inner lamellae. The latter appeared whorl-like due to a concentric arrangement of parallel membranes. Xanthophores contained two types of cytoplasmic vesicles, namely, small and large ones, which appeared hollow, electron-lucent or had slightly electron dense particles. Sections of some large vesicles revealed a very thin membrane enveloping these droplet-like vesicles. Large spherical melanosomes were highly electron dense in the cytoplasm of the melanophores. These cells also possessed slightly electron dense organelles which could be partially melanised pre-melanosomes. The iridescent sheen on the fish body was due to refraction and reflection from reflecting platelets in iridophores closely associated with other chromatophores. The designation 'irido-melanophore unit' is proposed for one such close association which comprises an iridophore located above a melanophore. Variable inclination angles and thicknesses of reflecting platelets are elucidated to cause the iridescent sheen. Thin platelets oriented at small angles to the long axis of a cell produce purplish-blue hues whilst thick and thin platelets at large angles generate a silvery-golden sheen.

Keywords Ultrastructure – erythrophores – xanthophores – melanophores – iridophores – *Betta splendens*

INTRODUCTION

Most teleosts have five basic types of pigment cells (chromatophores), which are classified as melanophores, erythrophores, xanthophores, iridophores and leucophores [1]. The *Xiphophorus* fish has a unique chromatophore, called the xantho-erythrophore, which has a yellow central region and red periphery [2]. Chromatophores can be separated into three main shapes: corolla with petal-like processes radiating from a disc-like centre, dendritic with irregular processes branching from a small cell body, and small rounded punctate cells with short stumpy processes [3].

Dark coloration is attributed to melanophores which have brown or black melanin pigments synthesised in granular spherical organelles called melanosomes [1]. Melanin is stored within Golgi- and endoplasmic reticulum-derived pre-melanosomes that develop into mature melanosomes [4]. Erythrophores contribute to red pigmentation in many fishes [1]. Matsumoto [5] and Matsumoto and Obika [6] identified these pigments in goldfish erythrophores as a mixture of drosopterin compounds enclosed within spherical cytoplasmic organelles designated as pterinosomes. Besides pterinosomes, erythrophores also contain carotenoid pigments as specific components for storage [1].

Yellow or xanthic (gold) coloration in fishes is attributed to xanthophores which contain carotenoids

[1]. These lipid-soluble carotenoid pigments are dietary in origin and stored in vesicles of different sizes among the pterinosomes. These vesicles possess an extremely thin membrane extending from or in direct contact with the endoplasmic reticulum [7]. Goodrich *et al.* [8] found zeaxanthin and lutein to be the carotenoids responsible for yellow coloration. Besides carotenoids, xanthophores also have yellow and colourless pteridines enclosed within pterinosomes. The xantho-erythrophore, found only in Xiphophorine fishes, is a single cell with carotenoids in its yellow centre and pteridines in its red periphery [2, 3].

In addition to brightly coloured chromatophores, there exist cells with colourless pigments and crystals. These are termed guanophores due to the presence of guanine in granular or crystalline form and as thin light-reflecting platelets [1]. Guanophores consist of iridophores and leucophores. Iridescence, silvery or metallic tones such as blue, turquoise, green and purple colours are caused by interference, refraction, reflection, diffraction and Tyndall scattering of light in iridophores [1]. Iridophores possess numerous flat vesicles, in each of which a thin reflecting platelet is embedded. These guanine platelets are regularly arranged in parallel stacks, forming an inclination angle with respect to the surface of scales and skin along the lateral plane of the fish [9]. Leucophores contain whitish granular leucosomes that are randomly oriented in the cytoplasm [1].

Fishes of the Family Belontiidae have been popular with aquarists for many years, primarily because of their varied array of pigmentation patterns. Previous investigations have concentrated on the genetic basis of fancy colour varieties and morphological mutants of the Siamese fighting fish, *Betta splendens* Regan [10-13]. However, there is a lack of investigations on the ultrastructural morphology of the chromatophores of this teleost species [3]. This study endeavours to clarify the cellular basis of coloration of the long-finned *B. splendens* using bright-field light and transmission electron microscopy (TEM) techniques.

MATERIALS AND METHODS

Red, Golden, Dark Blue and Turquoise varieties of long-finned *B. splendens* were obtained from a commercial farm in Lim Chu Kang, Singapore. The fishes were maintained as described by Khoo [3]. Scales from the dorso-lateral regions were detached and mounted individually in teleost physiological saline (TPS; 6.5g NaCl, 0.4g KCl, 0.15g $CaCl_2.2H_2O$, 0.15g $MgSO_4.7H_2O$ in 1 L deionised-distilled water, pH 7.3). An Olympus BHS-2 binocular light microscope (Tokyo, Japan) was used at 200-1,000× magnifications to observe the chromatophores.

Ultrastructures of the chromatophores were studied using a JEOL JEM 100CXII STEM transmission electron microscope (Tokyo, Japan) at 7,200-54,000× magnifications. Scales, detached from the dorso-lateral region of each variety, were immersed in TPS (pH 7.2, 224 mOsm). These scales were pre-fixed for 60 min in a 2.5% glutaraldehyde-2% paraformaldehyde mixture (TAAB Labs., UK) prepared in Sorensen's phosphate buffer (11.88 g $Na_2HPO_4.2H_2O$ and 9.08 g $KH_2PO_4.H_2O$ in 1 L deionised-distilled water, pH 7.2. Osmolarity was adjusted with sucrose to 224 mOsm using a WESCOR 5500 Vapour Pressure Osmometer) at 20-23°C. Subsequently, scales were washed repeatedly with Sorensen's buffer for 20 min.

Scales were post-fixed for 60 min at 20-23°C with 1% OsO_4 (TAAB Labs., UK), washed thoroughly in buffer for 20 min, dehydrated through an ascending ethanol (Merck, Germany) series (30-100%) followed by infiltration with a 1:1 propylene oxide (TAAB Labs., UK) and 100% ethanol mixture, and finally with 100% propylene oxide. A 1:1 mixture of propylene oxide and Spurr's low viscosity embedding resin (EM Sciences, USA) was used to enhance resin infiltration before the scales were finally embedded in Spurr's resin at 80°C in a Memmert oven (Schmidt Scientific, Germany) for 48-72 hr.

Ultrathin sections (80-120 nm) of the scales were cut on an LKB Ultrotome Nova (Bromma, Sweden) with newly prepared glass knives and mounted on formvar-coated SPI 100-150 mesh size copper grids (USA). The sections were dried in a desiccator, stained with uranyl acetate (TAAB Labs., UK) for 15 min, washed thoroughly with five changes of distilled water to remove excess stains, and double-stained with lead citrate (TAAB Labs., UK) for a further 15 min before a final rinsing with distilled water. Mounted, stained longitudinal sections of the scales were dried overnight in a desiccator and observed at an accelerating voltage

of 80-100 kV. Photomicrographs were taken at different magnifications using Kodak 4489 ESTAR film.

RESULTS AND DISCUSSION

Light microscopy and TEM studies reveal that chromatophores of *B. splendens* display morphological and ultrastructural features specific to each cell type. Erythrophores have discrete spherical red pterinosomes (Fig. 1A) [1, 5, 6]. According to Matsumoto [5], Matsumoto and Obika [6], and Valenti [2], the red pteridines in erythrophores can be separated into different drosopterin compounds. Conversely, the reddish-brown pigments in *B. splendens* erythrophores might be a pigment of non-pteridine origin [3]. This pigment might be an intermediate product from a blocked melanin synthesis pathway or phaeomelanins [8, 14].

The pterinosomes in erythrophores of *B. splendens* show large discrete oval-shaped cytoplasmic organelles with a trilaminar limiting membrane and whorl-like concentric internal lamellae of parallel membranes (Fig. 2A,B) [3]. These organelles have identical ultrastructures as the pterinosomes of *Xiphophorus helleri* [5], sailfin molly, *Poecilia latipinna* [15] and *Oryzias latipes* [7]. Some of these organelles appear similar to the pterinosomes of goldfish erythrophores [6] and medaka xanthophores [7] in having slightly electron dense particles and vesicular inclusions (Fig. 2B). These intracellular structures might represent various stages during pterinosome development to form mature organelles that possess well developed inner lamellae [7]. They are also comparable to the pre-melanosome vesicles that derive from the Golgi complex during melanogenesis [15].

Xanthic or golden pigmentation is dependent on carotenoids in *B. splendens* xanthophores [3]. The presence of carotenoid pigments in xanthophores has been reported previously in various fish species [1, 2]. Due to the dense yellow pigment, xanthophores appear diffused and do not show discrete margins under light microscopy (Fig. 1B). Ultrastructural details of xanthophores show two types of cytoplasmic vesicles (vacuoles), i.e., those

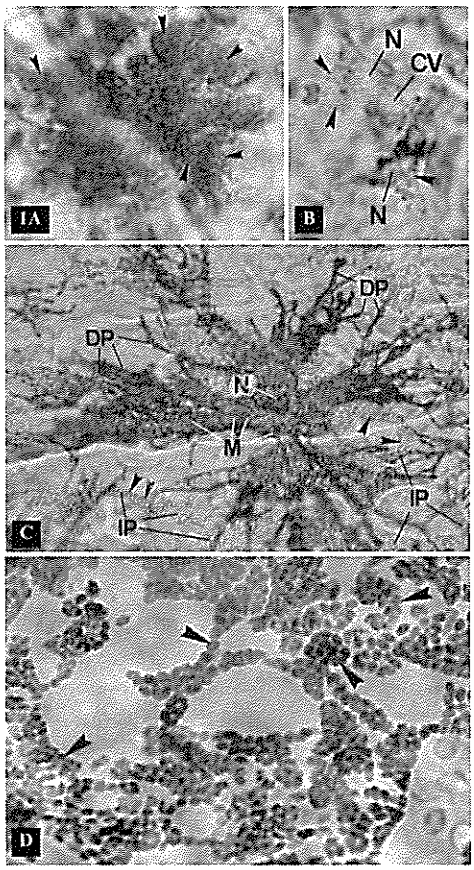

Figure 1. A – Corolla-shaped erythrophore of the Red variety with red pterinosomes (arrowheads) uniformly distributed in the cytoplasm (1,000×). **B** – Dendritic xanthophores of the Golden variety with yellow pterinosomes (arrowheads), carotenoid vesicles (CV) and colourless nucleus (N) (1,000×). **C** – Melanophore of Dark Blue variety having melanosomes (M), nucleus (N) and long dendritic processes (DP), with multicoloured iridophore plate-lets (arrowheads) in long inter-linking processes (IP) surround-ing the melanophore (1,000×). **D** – Multicoloured platelets (arrowheads) in iridophores that are interlinked to form a mesh-like network in the Turquoise variety (1,000×).

with large seemingly hollow electron-lucent vesicles (Fig. 2C), presumably containing carotenoids, and smaller ones which have slightly electron dense particles [3]. The large droplet-like vesicles seem enveloped within a very thin limiting membrane, and are apparently attached to endoplasmic reticular-like tubulo-vesicular structures. These vesicles appear empty possibly due to dissolution of lipid-soluble carotenoids during fixation and dehydration processes for electron microscopy [7]. Small electron-lucent vesicles interspersed among the large vesicles may possibly contain yellow and colourless pteridines [3]. Carotenoid vesicles of *B. splendens* seem identical to the vesicles in xanthophores of goldfish and medaka [5-7]. Under the TEM, xanthophores of *B. splendens* often have nuclei that are characteristically polymorphic (Fig. 2C).

Dusky pigmentation in *B. splendens* is attributed to dark, dense melanin pigment, bound within melanosomes in the cytoplasm (Fig. 1C). Under the TEM, vertically sectioned *B. splendens* melanophores reveal large spherical membrane-bound melanosomes in the cytoplasm (Fig. 2D) [3]. Mature melanosomes are highly electron dense due to their heavy melanisation. They are usually larger than pterinosomes but appear similar in size to carotenoid vesicles (Fig. 2D) [3, 4, 15].

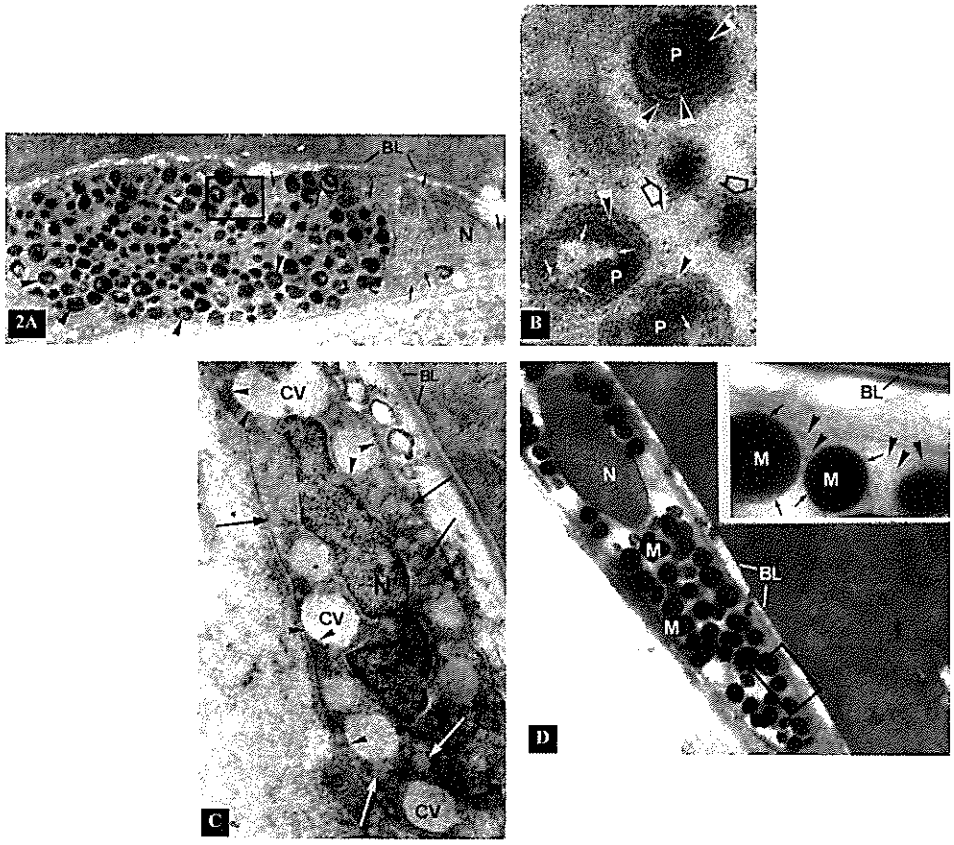

Figure 2. TEM micrographs. **A** – Erythrophore with a large nucleus, membrane-bound pterinosomes (arrowheads) and small electron-lucent vesicles (small arrows) (8,700×). **B** – Outlined portion of the erythrophore in (A) magnified to show pterinosomes (P) having whorl-like internal lamellae (arrowheads), and slightly electron dense particles (open arrows) and vesicular inclusions (small arrows) (54,000×). **C** – Xanthophore with large hollow carotenoid vesicles (CV) and smaller ones with slightly electron dense particles (arrows) together with a membrane-bound polymorphic nucleus below the basal lamina. A thin membrane (arrowheads) seems to envelop the large vesicles (21,000×). **D** – Melanophore with highly electron dense melanosomes (M) and a large nucleus (7,200×). Inset: Outlined region of the melanophore highly magnified to show membrane-bound (small arrows) melanosomes (M) (28,500×). (BL: basal lamina, N: nucleus)

These large flat melanophores usually have long dendritic processes and possess large centrally-located membrane-bound non-polymorphic nuclei (Fig. 2D). Numerous small and slightly electron dense ellipsoidal vesicles in some *B. splendens* melanophore sections might possibly be pre-melanosomes that later fuse and develop into mature melanosomes following melanin biosynthesis [3, 4, 15].

Silvery or metallic iridescence on the body and fins of *B. splendens* is contributed mainly by iridophores that are present as individual cells or closely associated with an underlying layer of melanophores and other chromatophores (Fig. 1C,D) [3]. Under bright-field light microscopy, iridophores are observed as large multicoloured granules or oval-shaped platelets having a range of purple, pink, yellow, white, blue and green hues (Fig. 1D). The platelets of *B. splendens* were reported to lack well-defined corners and had seemingly wavy or rippled alternating light and dark bands [12]. Light microscopy observations on iridophores of the neon tetra also revealed that these plates of guanine crystals are hexagonal in shape [9].

Metallic colours of Dark Blue and Turquoise *B. splendens* are a result of reflection from a melanophore screen beneath the iridophores (Fig. 1C), with combinations of melanophores and xanthophores imparting a wide range of blue and green hues as in the blue-green lateral stripes of the neon tetra [3, 9]. Besides reflection, white light is also refracted by iridophores and Tyndall-scattered by white and colourless guanine particles (leucosomes) in leucophores (Fig. 1E) [16]. The Tyndall effect produced by leucophores of the Golden *B. splendens* together with refraction from iridophores that interdigitate with yellow xanthophores might generate silvery-golden iridescence [3]. Lucas [12] suggested that the 'spread iridocyte colour' of metallic blue is a result of Tyndall blue when viewed against a dark screen of melanophores. Thus, xanthophores interspersed among melanophores and densely packed refractive iridophores might cause Tyndall blue to appear green in the Turquoise *B. splendens* [3, 9, 16].

Electron microscopy of iridophores yielded interesting results for the metallic coloured variants. The Golden variety has two types of membrane-bound reflecting platelets in iridophores, i.e., large thick platelets and small thin ones (Fig. 3A) [3]. These stacked platelets appear regularly oriented in parallel arrays at various oblique angles. The actual platelets are usually not observed but are represented by empty membrane-enclosed spaces as they might have been detached during sectioning and staining. In this study, some platelets were well preserved and appeared as thin lines enveloped within fine membranes (Fig. 3). These transparent platelets, inclined at varying angles, form a multilayered thin-film interference system in the liquid cytoplasm, hence rendering the cells very refractive and reflective [3, 9, 16]. Wallbrunn [11] found that iridescent blues and greens, for which *B. splendens* is famous, could be traced to two chromosomal loci whereby one locus affects the density of overlying iridophores, and the other the thickness of guanine platelets and the refraction of a particular colour.

The Dark Blue and Turquoise variants have iridophores situated above melanophores with cell processes that interdigitate and overlap (Fig. 3B-D). It is proposed that the designation 'irido-melanophore unit' be used to define the close association represented by these two pigment cell types of *B. splendens* [3]. This study reveals that the irido-melanophore unit comprises two cell types which form interdigitations with xanthophore processes (Fig. 3B). The irido-melanophore unit of *B. splendens* also parallels the melanophore-backed iridophores of the neon tetra [9, 16]. Reflecting platelets in the irido-melanophore units are also arranged in parallel arrays and enclosed within multiple stacks in the cytoplasm (Fig. 3) [3].

Different inclination angles and varying thicknesses of the iridophore reflecting platelets primarily generate the metallic iridescent hues of *B. splendens*. The inclination angles of reflecting platelets in the irido-melanophore unit appear to increase from the Dark Blue to the Turquoise and Golden varieties (Fig. 3) [3]. Calculated angles of platelet orientation in iridophores of the neon tetra reveal that dark violet hues are generated by very small angles while red iridescence is attributed to the largest [9, 16]. In essence, light of shorter wavelengths such as blue and violet are reflected by small angles but larger angles reflect those of longer wavelengths, e.g., yellow and red [16]. This supports Khoo's [3] observations in which platelets that are almost horizontally oriented generate the

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Editorial

Most of the articles in this issue are devoted to some aspects of life sciences. This is not surprising because of the current emphasis given to this field all over the world.

Such a focus is understandable given the potential of life sciences to reveal the ultimate scientific knowledge on life, and also their potential for wealth creation and sustaining societal well-being.

The paper on sustainable palm oil production is especially interesting as energy has become a global buzz word. The energy issue is given prime time in all kinds of fora, be they climate change, causes of political conflict, or biotechnology. It is particularly heartening, therefore, to read in Soh *et alia's* article that they believe the only economically feasible renewable and expandable source of oil which can significantly meet the demand for biodiesel is palm oil. They recommend committed and continuous efforts in R&D and innovation.

And this is what JOSTT is about: a platform where research for development and innovation are showcased. Hence we hope that the journal will continue to attract papers that outline the outcomes of fundamental research and demonstrate how scientific R&D can make a difference to solving society's problems.

Professor Datuk Dr. Mazlan Othman
Co-Chairman, Editorial Board

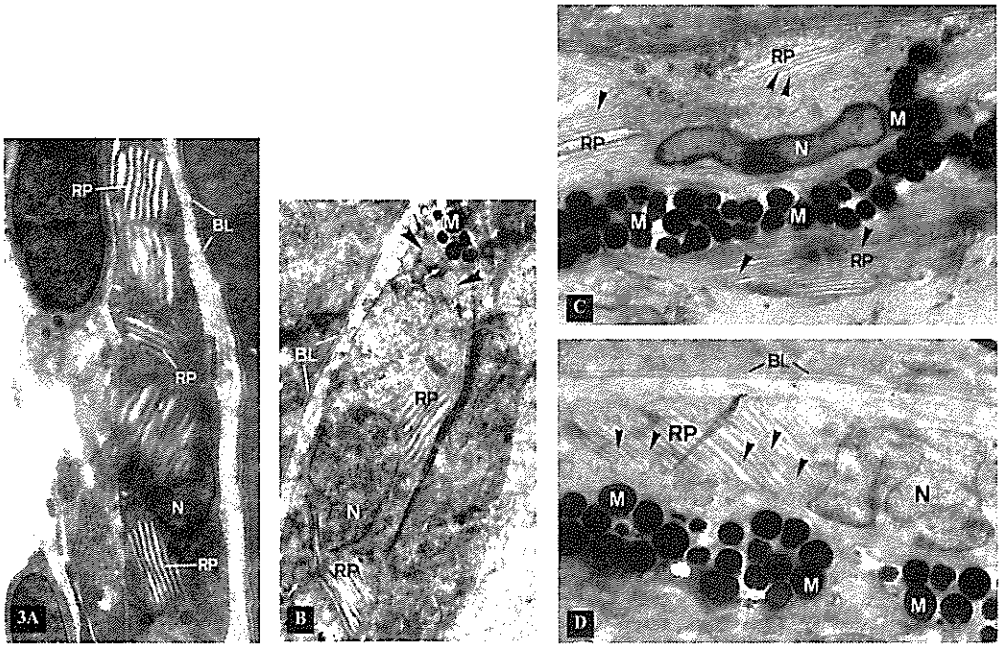

Figure 3. Electron micrographs. **A** – Iridophore with stacks of thick and thin reflecting platelets (RP) oriented at different angles to the basal lamina (10,800×). **B** – Three pigment cell types, i.e., an iridophore with stacks of thin reflecting platelets (RP), a xanthophore process with carotenoid vesicles (arrowheads) and a melanophore process with highly electron dense melanosomes (M) (7,200×). **C** – Cell process of a melanophore containing electron dense melanosomes (M) located in between iridophores with stacks of thin horizontally oriented reflecting platelets (RP) (7,200×). **D** – Iridophore with a polymorphic nucleus and reflecting platelets (RP) inclined at large angles to an underlying melanophore with electron dense melanosomes (M) (10,800×). Reflecting platelets are depicted as thin lines (arrowheads) in (C) and (D). (BL: basal lamina, N: nucleus)

purplish-blue sheen of the Dark Blue variant while large angles produce green and silvery-golden iridescence of the Turquoise and Golden varieties, respectively (Fig. 3).

In conclusion, this study has provided an in-depth description of the cellular basis for the brilliant colour patterns of the perennially popular long-finned Siamese fighting fish, *B. splendens*, and has clearly illustrated the specific combinations of pigment cells that are responsible for the tremendous range of colour variations of this teleost species [3]. Additionally, reflection, refraction and Tyndall light-scattering, coupled with varying inclination angles and thicknesses of guanine reflecting platelets in iridophores that are closely associated with other chromatophores, are inferred to produce a wide spectrum of metallic iridescent colours ranging from silvery-golden to dark blue in *B. splendens*.

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In vitro primary culture of erythrophores from fin explants of the Siamese Fighting Fish (*Betta splendens*)

Gideon Khoo[1], Lim Tit Meng[2] and Violet P. E. Phang[2]
[1]Faculty of Engineering & Science, Universiti Tunku Abdul Rahman,
Setapak, 53300 Kuala Lumpur, Malaysia
[2]Department of Biological Sciences, National University of Singapore,
Kent Ridge, Singapore 117543
(Email: gideonkhoo@mail.utar.edu.my)

Abstract Chromatophores are responsible for a myriad of animal colorations. The aim of this study was to establish a technique for long term in vitro culture of fish pigment cells. The fin explant culture system was employed to isolate erythrophores from the Siamese fighting fish (*Betta splendens*). Optimized pre-culture enzymatic treatment of caudal fin explants consisted of 0.3% collagenase and 0.2% trypsin for 60 min at 20°C. Erythrophores migrated out of the explants and formed cell colonies after 2 weeks of culture. A rich culture medium comprising Leibovitz's L-15 supplemented with 20% heat-inactivated foetal bovine serum, 1% carp (*Cyprinus carpio*) serum and 1% penicillin-streptomycin was effective in maintaining the cultures for at least 90 days. These cells survived best at ambient CO_2 levels of 0.03-0.05%. Mitotic activity was not detected despite treatment with mitosis-inducing agents, e.g., insulin and epidermal growth factor. In vitro cultured erythrophores displayed two main morphological shapes, namely, flattened corolla or discoidal, and those with long dendritic cytoplasmic processes. A significant number of cells became multi-nucleated after 30 days of culture. Some erythrophores and colourless cells (possibly melanoblasts and fibroblasts) became darkly pigmented due to melanisation and differentiation, respectively.

Keywords erythrophores – primary culture – fin explants – *Betta splendens*

INTRODUCTION

In recent years, success in the development of fish cell culture has significantly contributed to the establishment of various continuous cell lines from coldwater and warmwater teleost species for studying cytogenetics, physiology, biochemistry, differentiation, metamorphosis, malignant transformation, carcinogenesis and fish virology [1-6]. These cell lines, comprising epitheloid and fibroblastoid monolayer cultures, have been isolated and developed from tissues of economically important fishes and elasmobranchs [5-8].

In the past three decades, melanophores, erythrophores and iridophores have emerged as classical cell models for examining the mechanisms of pigment organelle motility [9, 10]. Although much has been learnt about the effects of hormones and ions on dermal pigment cells, these studies have been restricted to cells located only on the scales and in the skin. However, a unique approach to study the roles played by the cytoskeleton during pigment translocation has led to the successful isolation, purification, establishment and culture of swordtail erythrophores [1], xanthophores of goldfish [11, 12], and melanophores of black moor goldfish [13] and *Oryzias latipes* [9, 14].

In addition to terminally differentiated chromatophores, studies have been conducted using cell lines developed from neoplastic pigment cells, e.g., goldfish erythrophoromas [2-4]. These highly proliferative tumourous cell lines have been chemically induced to form melanized clones with different phenotypic characteristics and are able to undergo multiple differentiation in the presence of autologous fish serum [15].

The aim of this study was to establish the optimal conditions for *in vitro* primary culture of

erythrophores from fin explants of the Siamese fighting fish, *Betta splendens*. Erythrophores of *B. splendens* were chosen due to their highly intense blood-red coloration and the ability of these cells to rapidly change colour intensities [16]. This study proposes a novel cell culture model for morphological, physiological and biochemical investigations on the mechanisms that control pigment granule transport. Furthermore, these cultures may permit the induction of permanent and neoplastic *B. splendens* cell lines.

MATERIALS AND METHODS

Only the Red *B. splendens* variety was used as the yield and purity of erythrophores were higher than in other variants. The source and culture of this fish were as described by Khoo [16]. The procedures established by Obika [9], Matsumoto *et al.* [1], Lo *et al.* [11], Clark *et al.* [13], and Chen and Kou [5] were modified by Khoo [16] to optimise the culture conditions for *B. splendens* erythrophores. Fins were dissected from the fish, sterilised in 70% ethanol and rinsed 3-4× with sterile teleost physiological saline (TPS). Fins were minced into small fragments and incubated in sterile dissociation medium (DM; 0.25% collagenase Type 1A and 0.20% trypsin in TPS [Sigma, USA]) for 60-90 min with 150-180 rpm agitation at 20-23°C on a Certomat M Orbital bench-top shaker.

After dissociation, fin fragments were stripped of epidermis with fine forceps, rinsed 4-5× in sterile TPS and further dissected into smaller pieces in sterile culture medium comprising Leibovitz's L-15 medium with 2.05 mM L-glutamine supplemented with 20% foetal bovine serum (FBS) (L-15 and FBS from GIBCO & JRH Biosciences, USA) and a 100 IU/ml penicillin G - 100 µg/ml streptomycin mixture (Sigma, USA). FBS was pre-heated at 56°C for 30 min to inactivate proteases [8, 17]. All solutions were sterile-filtered through Corning 0.22 µm cellulose acetate bottle-top filters (USA). Glassware and metal instruments were autoclaved in a HA-300MII Automatic High Pressure Steriliser (Hirayama, Japan) for 40 min at 125°C and 1.5 kg/cm^2 pressure. All experimental phases were conducted under sterile conditions in a HFL-120 Laminar Air Flow WorkStation (Gelman Sciences, Australia).

Fin fragments (10-20 pieces/well) were seeded onto γ-irradiated sterile collagen-coated 24-well plastic Nunclon Delta Multidish (Denmark) and Falcon culture dishes (Becton-Dickinson, USA). Prior to fin explantation, culture dishes were coated with calfskin collagen Type I (Sigma, USA). Solid form collagen was dissolved in 0.1% acetic acid (Merck, Germany) in distilled-deionised autoclaved water at 4°C for 48 hr to prepare a 100% stock solution which was further diluted 10 times with distilled autoclaved water. Subsequently, 1 mL of diluted collagen solution was pipetted into each well, air-dried in the laminar flow chamber for 60 hr and sterilised by UV-irradiation for 2 hr prior to storage in UV-irradiated plastic bags, or used directly for cell culture.

Fin explants with erythrophores were cultured on collagen-coated plastic culture dishes with 1 mL culture medium/well at two CO_2 levels: 0.03-0.05% (ambient condition) and 5%, in a Queue 2700 Water-Jacketed CO_2 Incubator (USA) at 20-23°C with sterile-filtered atmospheric air. The culture medium was renewed every third day. Another series of cultures was set up with addition of 1% common carp (*Cyprinus carpio*) serum together with the above-mentioned controls without serum. Carp serum was obtained by caudal vein-puncture [16]. Blood was drawn using 3 mL Monoject sterile disposable syringes (Sherwood Med. Ind., USA), centrifuged at 10,000 rpm for 15 min at 4°C in a Beckman J2-21 centrifuge (USA) with JA-20 fixed angle rotor, and stored in a –85°C Queue deep-freezer (USA).

Cultures were observed and photomicrographed over 90 days using an Olympus IM inverted light microscope (Japan)

with a C-35AD2 camera attachment and PM-10ADSP Exposure Unit. Effects of the CO_2 level and carp serum supplementation on erythrophore migration, attachment, maintenance, morphology and proliferation were qualitatively compared to determine the optimal culture conditions.

RESULTS AND DISCUSSION

This study demonstrates that, through the approach of fin explantation, erythrophores of *Betta splendens* could be cultured under conditions suitable for morphological and biochemical studies. Although melanophores, erythrophores and xanthophores of teleosts have been isolated and cultured [1, 9, 11], these procedures involve cell dissociation from scales and fins followed by cultivation through differential adhesion to the substrata. These techniques proved unfeasible for erythrophores of *B. splendens* [16]. The first method caused extensive shearing of cell dendrites while the second adversely affected the cells, resulting in cell lysis and death within a few days after centrifugation.

In the procedure reported here, fin explants of Red *B. splendens*, which contained mainly erythrophores, were seeded onto collagen-coated culture plates to induce erythrophore migration. Collagen, an extracellular matrix component in tissues, was used to promote cell adhesion and plating while maintaining the morphological appearance and shapes of these cells [1, 14, 17]. Iwata *et al.* [14] reported that shapes of melanophores of the medaka were regulated by fibronectin in collagen-coated substratum since fibronectin binds specifically to collagen and cells adhere to the latter *via* fibronectin. In contrast, *B. splendens* erythrophores, cultured on only collagen substratum (Figs. 1-4), showed discoidal and dendritic morphology similar to cells *in vivo* [16]. This is unlike melanophores of the medaka which require a specific fibronectin-collagen concentration before they could spread and adhere to the substrate [14].

Plain uncoated dishes used as controls did not permit the culture of erythrophores beyond a week as the cells did not migrate from the fin pieces and remained punctate in shape [17]. Fin fragments and cells also lacked stable anchorage and were easily detached from uncoated dishes [1]. Fukuzawa *et al.* [17] observed that, among the extracellular matrices, collagen type I did not affect pigment cell expression while fibronectin inhibited the expression of cultured melanophores and moderately stimulated iridophores and xanthophores. In addition to providing a substrate for cell migration and adhesion, Iwata *et al.* [14] and Fukuzawa *et al.* [17] indicated that these extracellular matrices did not influence the expression of terminally differentiated chromatophores but might play a role in the differentiation of neural crest cells. Cell division seemed absent in the *B. splendens* erythrophore cultures although a number of cells were bi- or multinucleated (Figs. 1-4) [16]. This was also observed in the cultured erythrophores of swordtails [1], melanophores of black moor goldfish [13] and goldfish erythrophoromas [2, 15].

Fin fragments were seeded at high densities of 10-20 per well to ensure that intrinsic growth factors released from explants could facilitate cell adhesion onto culture dishes and maintenance of erythrophores *in vitro* as reported for melanophores of black moor goldfish [13]. Autologous carp serum also contains growth factors besides nutrients that are required to prolong the lifespan and retain the pigmentation in cultured pigment cells [3, 4, 15]. This was verified by Khoo [16] who reported the increased lifespan of *B. splendens* erythrophores cultured in media supplemented with carp serum (Figs. 1-4).

Erythrophores of *B. splendens* were cultivated in Leibovitz's L-15 culture medium using two CO_2 levels with and without 1% carp serum supplementation [16]. Twenty percent of FBS was used with carp serum to enhance expression and attachment of cells. FBS is a routine supplement for amphibian cell culture and its usage has been extended to the primary culture of fish cell lines [1, 2, 8, 15, 17]. Leibovitz's L-15, a general medium for poikilotherm cell culture, was employed instead of the more common Eagle's Minimum Essential Medium (EMEM), Opti-MEM I, Ham's F-10 and Medium 199, as L-15 is well suited for supporting and maintaining cell growth in non-CO_2 equilibrated conditions [16]. Moreover, addition of sodium bicarbonate is not required with L-15 as the cultures need not be performed at high CO_2 levels [2, 5, 8]. Five percent of CO_2 was used in this study

to investigate its effects on the erythrophores of *B. splendens* [16], an anabantoid well suited to life in small pools of stagnant and polluted water with low O_2 levels.

B. splendens erythrophores exhibited discoidal and dendritic shapes which were dependent on the culture conditions (Figs. 1-4) [16]. The cells had extremely long and thin dendrites at 5% CO_2 with and without carp serum (Figs. 1, 2). This 'superdendritic' morphology was also noted by Iwata *et al.* [14] for medaka melanophores. Most superdendritic erythrophores in 5% CO_2 without carp serum had very small cell bodies (Fig. 1). These cells failed to attach to the collagen substratum and did not show flat disc-shaped configuration nor survived beyond 28 days [16]. Conversely, for experiments in which 1% carp serum was added, the cells showed extensive migration patterns and discoidal plating configurations (Fig. 2). The longer 60-day culture duration suggests enhanced cell-to-substrate attachment and cell maintenance when carp serum is present [16]. Stronger adhesion of erythrophores to the substrate is indicated by the formation of flat disc-like configurations and reduction of superdendritic morphology (Fig. 4).

Adhesion and maintenance of erythrophores

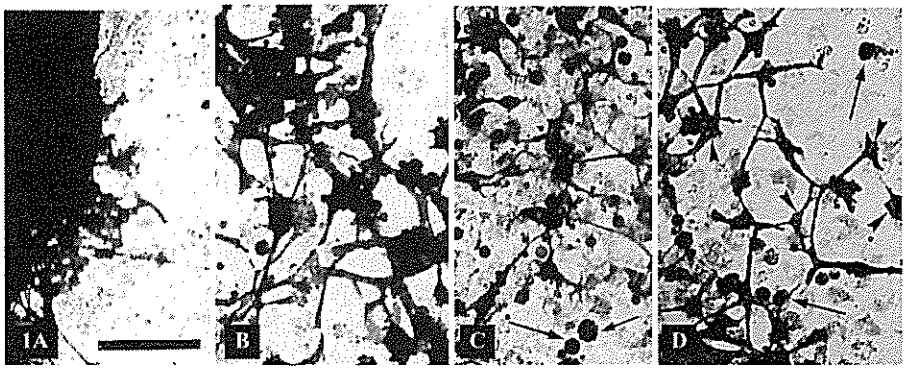

Figure 1. Photomicrograph of erythrophores cultured in 5% CO_2 without carp serum over a period of 28 days. Day of culture after fin explantation: **A**, 2; **B**, 11; **C**, 20; **D**, 28. **A-D** show non-extensive migration, sparse distribution, absence of cell division and death of cells. A large number of cells were punctuate-shaped (arrows) or lysed during culture. Dendritic cells had long thin processes and small cell bodies. Some cells were bi- or multi-nucleated (arrowheads). Magnification: **A**, 100×; **B**, 400×; **C**, 100×; **D**, 200×.

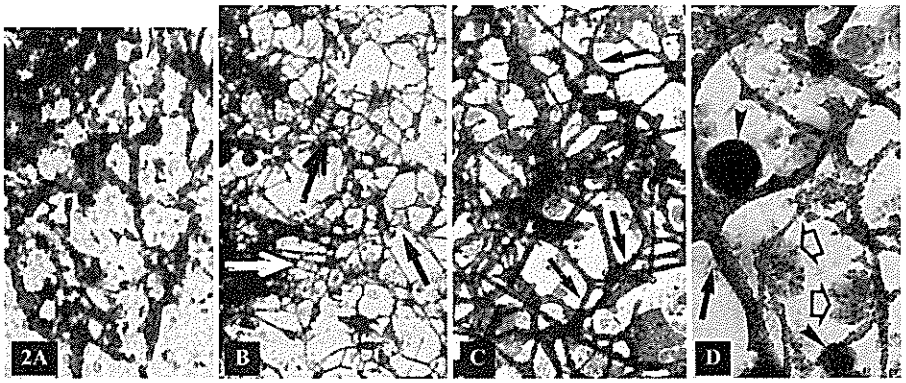

Figure 2. Erythrophores cultured in 5% CO_2 with addition of 1% carp serum for 60 days. This series of cultures is shown at 2 (**A**), 22 (**B**), 48 (**C**), and 60 (**D**) days after seeding of fin fragments. **A-D** depict extensive migration patterns, increasingly populated colonies, non-proliferation, absence of cell division, and subsequent cell death. Punctate-shaped cells (arrowheads) and also lysed ones (open arrows) were observed over time. Migrating cells were predominantly dendritic (white and black arrows) and these migrated over plated corolla-shaped ones throughout culture. Magnification: **A**, 200×; **B**, 40×; **C**, 400×; **D**, 600×.

cultured with and without carp serum were also enhanced by ambient CO_2 level as only a few cells were punctuate-shaped or lysed (Figs. 3, 4). Most of these cells had short broad dendrites, large cell bodies and flattened disc-like corolla shapes at low CO_2 levels, while migrating cells possessed long dendrites and medium-sized cell bodies not unlike the superdendritic configurations [16]. Optimal conditions were indicated by the 90-day culture duration in 0.03-0.05% CO_2 with carp serum (Fig. 4). These cells formed monolayer colonies that attained confluence after 30 days although continuous migration from fin explants occurred until this series was terminated after 100 days [16].

An almost homogeneous erythrophore culture was obtained in the presence of carp serum under optimal conditions after a month (Figs. 2, 4). Other chromatophores such as xanthophores and iridophores were not detected in the cultures [16]. Iridophores might be absent due to their inability to migrate from fin explants or they could have perished during the initial stages of fin preparation. Furthermore, iridophore expression might have been inhibited by FBS [17]. Using Khoo's [16] protocol,

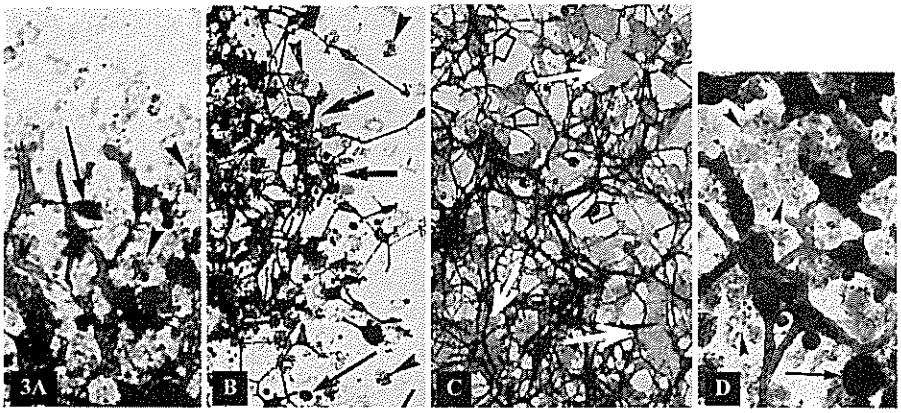

Figure 3. Erythrophores cultured for 48 days in 0.03-0.05% CO_2 without carp serum. This culture series is shown at 2 (**A**), 18 (**B**), 35 (**C**), and 48 (**D**) days after fin explantation. **A-D** show widespread migration and dense distribution patterns, absence of cell division and progressive cell death over time. Punctate-shaped (arrowheads) and lysed cells (open arrows) were observed in all culture dishes, but most of the cells were dendritic (short arrows). These migrated over disc-shaped plated cells (white arrows) which appeared only after the 35th day. Magnification: **A**, 200×; **B**, 40×; **C**, 150×; **D**, 600×.

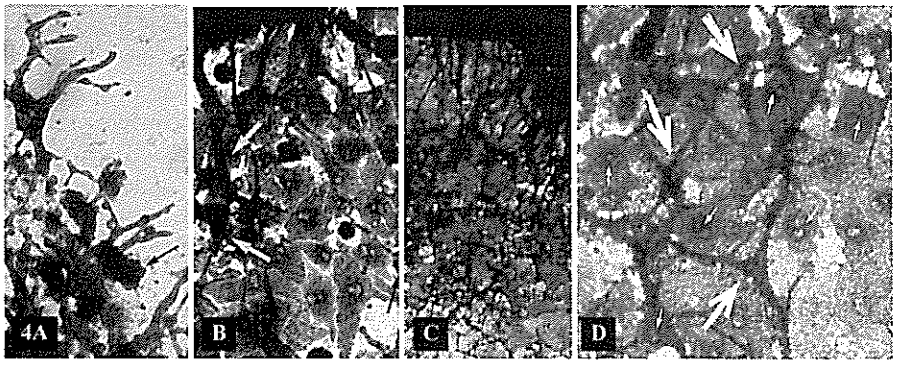

Figure 4. Erythrophores surviving for a 90-day culture period in 0.03-0.05% CO_2 with 1% carp serum supplemented culture medium. This culture series is shown at day 2 (**A**), 40 (**B**), 70 (**C**), and 90 (**D**). **A-D** show extensive cell migration from fins, absence of cell proliferation or growth, dense distribution patterns and successful maintenance over the culture period. **B-D** depict mainly disc-shaped plated cells (arrows) with some forming a confluent monolayer after a month. There were also some punctate (arrows) and lysed cells (arrowheads). Migrating cells were mainly dendritic-shaped (large white arrows) with long processes. Some cells were bi- or multi-nucleated (small white arrows). Magnification: **A**, 200×; **B**, 300×; **C**, 40×; **D**, 400×.

the major cell contaminants were fibroblasts and derivatives from cellular and acellular debris following fin digestion to remove the epidermis and expose the erythrophores. It was not possible to produce 100% pure erythrophore cultures as these terminally differentiated cells divided very slowly or not at all. Contamination by rapidly dividing fibroblasts also led to overgrowth of some cultures by these cells [16]. This can be reduced by regular passaging and selection of the required cells [11].

Multi-layered colonies of brown pigmented cells were also observed to form around the fin explants [16]. Some cultures, especially those exceeding 30 days at ambient CO_2 levels with carp serum, had disc-shaped cells containing brown or grey pigment granules that were morphologically similar to melanosomes [16]. These darkly pigmented cells appeared melanized and are presumed to be erythrophores that had undergone melanogenesis. These observations agree with findings that goldfish erythrophoromas could become melanized [15]. Cells from *B. splendens* fin explants might also comprise undifferentiated stem cells and unpigmented melanoblasts that could differentiate into melanized cells and melanophores [4, 12, 16].

In summary, this study describes a procedure for long-term primary culture of *B. splendens* erythrophores [16]. This novel cell culture model is proposed as a functional system for studying pigment granule movement, cell adhesion, plating configurations and cell morphology, as well as pharmacological analyses, growth regulation, proliferation and induction of neoplastic cell lines.

Acknowlegements – This work was supported by a grant from the National University of Singapore.

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Defense mechanisms of Malaysian termite soldiers

Chuah Cheng Hock
Department of Chemistry, University of Malaya, 50603 Kuala Lumpur, Malaysia
(E-mail: chchuah@um.edu.my)

Abstract The defense mechanism of Malaysian termite soldiers is reviewed. The unusual mechanical weapons, the use of chemical substances in defense, a combination of chemical and mechanical mode of defense, and the monophyletic regression of soldier mandibles concurrent with the evolution of chemicals are presented. The primitive soldiers depend solely on mechanical weapons for defense whereas the evolutionary advanced soldiers are characterized by conical, nozzle-shaped projections from which they eject chemical secretions that are produced by the enlarged frontal glands located within the head capsules. The chemical secretions consist of monoterpenes and diterpenes.

Keywords Soldier termites – chemical defense – frontal gland – terpenes – Isoptera

PERSPECTIVES AND OVERVIEW

There is a notable growth in the literature on the chemical defense secretions of termite soldiers in the last decade. Although the chemistry of insect secretions, especially of the termites, has grown tremendously, the ethological and morphological aspects of termites are less studied. The development of scanning and transmission electron microscopy has allowed a close-up view on the frontal defense glands of the termite soldiers. The ultra-fine structure of the salivary and frontal glands of some soldier castes including the bulbous part of the mandible, sensory bristles and glandular opening has been reported [1]. The defense of the colony is usually the responsibility of the specialized soldier caste which involves a sophisticated and peculiar defense mechanism. However, in some species, the colony is defended by the workers (soldierless termites of Africa - *Speculitermes*) by a defecating mechanism [2]. A peculiar behavioral defense by the worker caste in rapid nest-repairing to seal off entry points was noted for the Malaysian species *Globitermes sulpherus* and the unique pellet-plugs by *Prohamitermes mirabilis* [3,4].

Table 1. Defense strategies of termites.

Soldiers		Workers
Mechanical	Chemical	Behavioral
Plugging holes	Frontal glands	Well fortified nests
Biting mandibles	Salivary glands	Nest repair
Flicking mandibles		Sealing off entry points

DEFENSE MECHANISM OF MALAYSIAN TERMITE SOLDIERS

In Peninsular Malaysia, about 180 termite species have been recorded [4], representing three families and 42 genera. The defense strategies of Malaysian termites are shown in Table 1. Mechanical defense is associated with primitive termites whereas the evolutionarily advanced termites utilize chemical defense. The evolution of sophisticated methods of defense has led to termite soldiers being labeled as 'a caste of nightmarish monsters' [5], aptly verified by a cursory examination of the morphology of some selected Malaysian species. The defense mechanisms of termite soldiers from the Malaysian region can be classified into three distinct principal modes. They are mechanical, chemical and a combination of these modes. The mechanical mode in the defense of the colony would include plugging holes, biting and snapping action by the termite soldiers (Table 1). The chemical mode of defense is achieved by ejecting chemicals through the frontal gland or through the salivary gland.

The combination mode normally involves biting or snapping action with the simultaneous ejection of greasy, irritating, toxic or viscous chemicals.

Phragmosis

The prototypical phragmotic head of the soldier belonging to the genus *Cryptotermes* is well-adapted for plugging entry holes to the colony. The cylindrical head, a thick concave rostrum coupled with the short mandibles is specially designed to plug the entry holes thereby preventing the predators from entering the colony. Our observations so far indicated no chemical secretion is involved in the defense by *Cryptotermes cynocephalus*. There are few holes on the surface of the dry wood suggesting that only a small number of soldiers are needed in defense of the colony. This low soldier numbers with no surface foraging by the workers form a kind of static warfare.

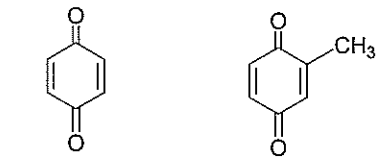
Mandibular biting

Mechanical defense that is activated by biting action (mandibles) alone can be illustrated by the genera *Glyptotermes* and *Neotermes* of the family Kalotermitidae. The soldiers' mandibles are serrated and the amplitude of the biting motion is small which is designated for the crushing action. Our observations of *Glyptotermes pinangae* and *Neotermes tectonae* from the Malaysian region indicate that no chemical secretion is involved in the defense of the colony.

Mechanical and chemical

The combined strategy of biting with the simultaneous secretion of chemicals through the salivary glands can be illustrated by the genera *Macrotermes*, *Odontotermes*, *Microtermes* and *Hypotermes* of the subfamily Macrotermitinae. Termite soldiers employing this mode of defense have well-developed sharp mandibles which are coupled with a well-defined chemical delivery system. Several *Macrotermes*, *Odontotermes*, *Microtermes* and *Hypotermes* soldiers found in the Malaysian region are equipped with large biting mandibles and in addition, are capable of secreting a small amount of chemicals through the salivary

Six species of the Macrotermitinae genera, viz. *Microtermes*, *Macrotermes*, *Odontotermes* and *Hypotermes* were examined by Tho and co-workers for the presence of defense secretions [4]. When provoked, five of the species released a defensive fluid from the big labial glands which was identified to consist mainly of benzoquinone and toluquinone. The defense secretions from *Microtermes globicola*, *Odontotermes redemani*, *Odontotermes praevalens*, and *Odontotermes horni* consist mainly of toluquinone. Benzoquinone was not detected in these species but was present in *Hypotermes obscuriceps*. Both the benzoquinone and its methyl homologue were detected in *Macrotermes carbonarius* but suprisingly, quinones were absent in *Macrotermes gilvus* [6].



Benzoquinone Toluquinone

The defense strategy by biting with the simultaneous ejection of chemicals through the frontal glands is illustrated by the genus *Coptotermes* from the subfamily Coptotermitinae. In *Coptotermes curvignathus* found in the Malaysian region that have smaller slender, incurved mandibles well-adapted for slashing action, the soldiers are capable of releasing a larger amount of defense chemicals. When provoked, *Coptotermes curvignathus* soldiers eject from the wide triangular openings of the frontal glands large droplets of white latex-like fluid. This fluid is well-suited to immobilize the small assailant because it oxidizes quickly, thereby entangling the enemies. Chemical analysis has shown that it is a suspension of saturated *n*-alkanes (C_{22}-C_{27}) in an aqueous solution of mucopolysaccharides of glucosamine and glucose units.

The dimorphic soldiers of *Schedorhinotermes* and monomorphic soldiers of *Parrhinotermes* and *Prorhinotermes* (Rhinotermitinae) have developed large tongue-like labra with a frill of short hairs at the distal end. These are used like brushes to spread the chemical secretions as they flow out from the fontanelle. The frontal secretion of *Prorhinotermes flavus* is toxic and is mainly composed of nitro-alkenes [7]. This nitro-group bearing compound such as *trans*-1-nitro-1-pentadecene was reported for *Prorhinotermes* from Panama region [8].

$CH_3(CH_2)_nCH=CHNO_2$

Nitroalkenes

Parrhinotermes may be considered the most primitive genus of Rhinotermitinae and the soldiers are monomorphic with the labral end exhibiting a well-spined daubing brush. The chemical secreted by *Parrhinotermes pygmaeus* and *Parrhinotermes aequalis* consist of a mixture of vinyl ketones [7].

$CH_2=CH(CH_2)_nC(=O)-CH=CH_2$ $CH_3(CH_2)_nC(=O)CH=CH_2$ $CH_3(CH_2)_nC(=O)CH_2CH_3$

Vinyl or ethyl ketones

The *Schedorhinotermes* soldier is capable of biting with its sharp mandibles and if this does not deter the enemy, a defense fluid [a mixture of vinyl ketones (alkanones, alkenones and alkadienones)] is secreted from the frontal glands [7]. This fluid flows along a medium groove to the tip of the labrum, resulting in 'daubing brush' action on the body of a small assailant with toxic and irritating chemicals. The well-developed 'daubing brush' of *Schedorhinotermes* has an advantage over *Parrhinotermes* and *Prorhinotermes* because it helps to speed up the rate of evaporation of the volatile components of the secretions due to the large surface area involved. The scent of the volatile components from *Schedorhinotermes malacensis* serves as chemical signal to summon nest mates to converge at the site of danger. Furthermore, the scent serves as a warning or repelling signal to a potential enemy.

The combined strategy of snapping with the simultaneous ejection of the chemical secretions through the frontal gland can be illustrated by the soldiers of subfamily Terimitinae. To accommodate such a mode of defense, the soldier mandible is twisted and it is well-adapted for snapping and flicking. The twisted mandible can also serve to block the enemy from entering the colony. If this does not deter the enemy from attacking the colony, a small amount of chemical is released to serve as a warning signal to the enemy. Termites possessing such features are the genera *Homallotermes*, *Procapritermes* and *Termes* of the subfamily Termitinae. *Homallotermes foraminifer* and *Homallotermes eleanorae* soldiers, with their symmetric mandibles, are capable of a slashing and flicking action. At the same time, a mango-smelling monoterpene, identified as terpinolene, is ejected at the predator. The soldiers of the cryptic termite *Termes comis* possess long and slender mandibles utilized for slashing and flicking action. This mode of defense is followed by secreting a small amount of the volatile monoterpenes hydrocarbons to repel the predators.

Similarly, *Procapritermes* soldiers with their twisted asymmetric mandibles can perform a snapping action and simultaneously a small amount of defense chemical is released through the frontal gland openings. This phenomenon was noted for the Malaysian *Procapritermes magnus*. *Pericapritermes speciousus* soldiers of the subfamily Termitinae have well-developed, twisted asymmetric mandibles for blocking entrances thus preventing assailants from entering the colony. An effective defense can be maintained by the release of chemicals through the salivary glands. The volatile chemicals serve to warn the enemy of the danger of penetrating the colony whereas the sticky fluid serves to immobilize the predator [9].

Autothysis and abdominal dehiscence

The genus *Globitermes* of the subfamily Termitinae possesses sharp biting mandibles and the chemical secretion is released by autothysis. This mode of operation by abdominal dehiscence occurs when the abdominal wall bursts as a result of vigorous defecation during defensive effort. The soldiers of *Globitermes sulfureus* have partial yellow abdomen and the mandibles are curved into an almost semi-circle that is well-adapted for slashing action. The species found in the Malaysian region has slightly smaller mandibles than the African species but it can release a large amount of defensive chemical by rupturing the salivary glands. This is accomplished due to the voluminous reservoirs of chemical secretion that extends right into the front half of the soldiers' abdomen. In the case of the large soldiers of *Pseudacanthotermes spiniger*, the reservoirs occupy 90% of the abdomenal cavity [10].

During combat, convulsive abdominal contraction activated by biting the predator is known to cause *Globitermes* soldiers to undergo

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autothysis, thus sacrificing themselves in the defense of the colony. This peculiar mode of defense results in threads of entangling chemical secretions that causes a viscous congealing liquid to be deposited on the bodies of the assailants. Observation also bears out this point, for when the colony of the elongated mound builder *Globitermes* is provoked, the soldiers will attack and bite the intruding object with their sharp mandibles. The bite may not cause a serious wound to humans; however if the action is followed by the rupturing of the abdomen of the termite body, there will be a deposition of a yellowish viscous liquid on the assailant or object. This peculiar suicidal behaviour was also observed in *Anoplotermes* and most Apicotermitinae [2]. Little is known of the chemistry of the secretion because the secretion undergoes rapid changes becoming opaque and sticky.

Chemical defense

The third principal mode of defense is purely chemical warfare. This mode of defense can be effected without actual physical contact with the enemy. The chemical is ejected through the well-developed elongated frontal gland. Most of the termite soldiers employing this mode of defense have evolved elongated pear-shaped heads while the mandibles have regressed to be non-functional. Soldiers employing this mode of defense are the genera *Longipeditermes* [11], *Bulbitermes* [12], *Nasutitermes* [13], *Havilanditermes*, *Leucopitermes*, *Lacessititermes*, *Subulioiditermes*, *Hirtitermes* and *Hospitalitermes* [14,15]. The soldiers employing this advanced mode of defense do not have salivary glands. The defense chemicals consist mainly of monoterpenes and diterpenes (Fig. 1). The volatile monoterpenes showing certain degree of irritancy and toxicity can serve as repellent against potential predators. The gluey diterpenes that dissolve in the volatile monoterpenes result in a hydrophobic interaction between the diterpenoid molecules and the monoterpene hydrocarbons. This hydrophobic interaction can prolong the rate of evaporation of volatile monoterpenes that serve as an effective

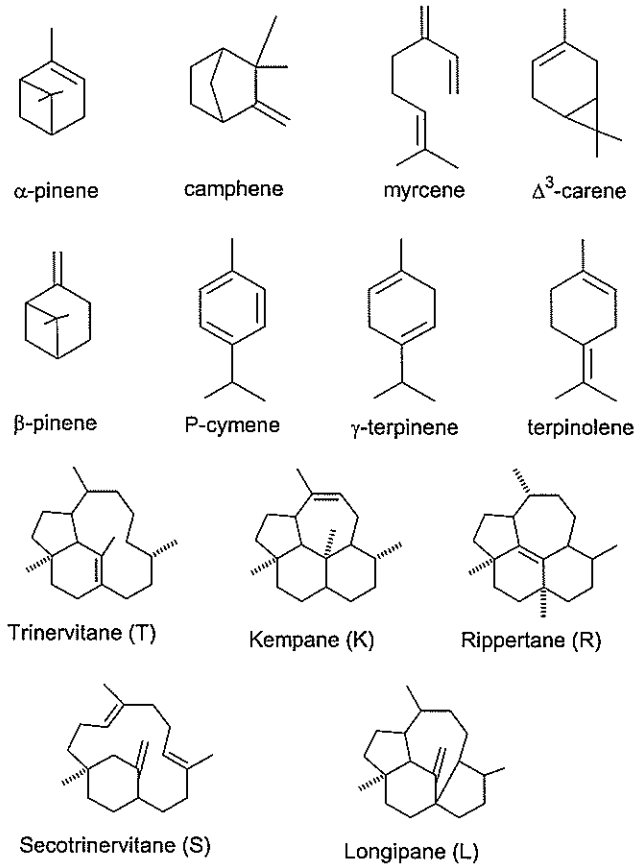

Figure 1. Defense chemicals of termites soldiers.

repellent agent. Finally, this viscous glue-like secretion when squirted onto the assailant's body results in the formation of a resin that immobilizes the predator.

The evolution of defense secretion with the concurrent regression of the soldier mandibles is illustrated in Figure 2. The genus *Longipeditermes* occupies an intermediate position in the evolution of Nasutitermitinae, and is among the first Nasutitermitinae to evolve the ability to manufacture diterpenes for defense. Structural variety of the diterpenes in the primitive glue-squirter is noted. However, the defense chemicals of the more evolved soldiers of *Leucopitermes* and *Havilanditermes* consist more of the trinervitane skeletal types. The evolutionary forces driving the nasute secretion toward a reliance on a limited repertoire of structure are unknown. They may include selection imposed by predation, or apparent speciation resulting from recognition of inter-colonial chemical differences and eventual reproductive isolation of the genetically homogeneous types. Although the chemical nature of nasute secretions has become well-studied, the underlying question of the biogenesis of the secretion and its importance in nasute ecology and evolution are as yet unanswered.

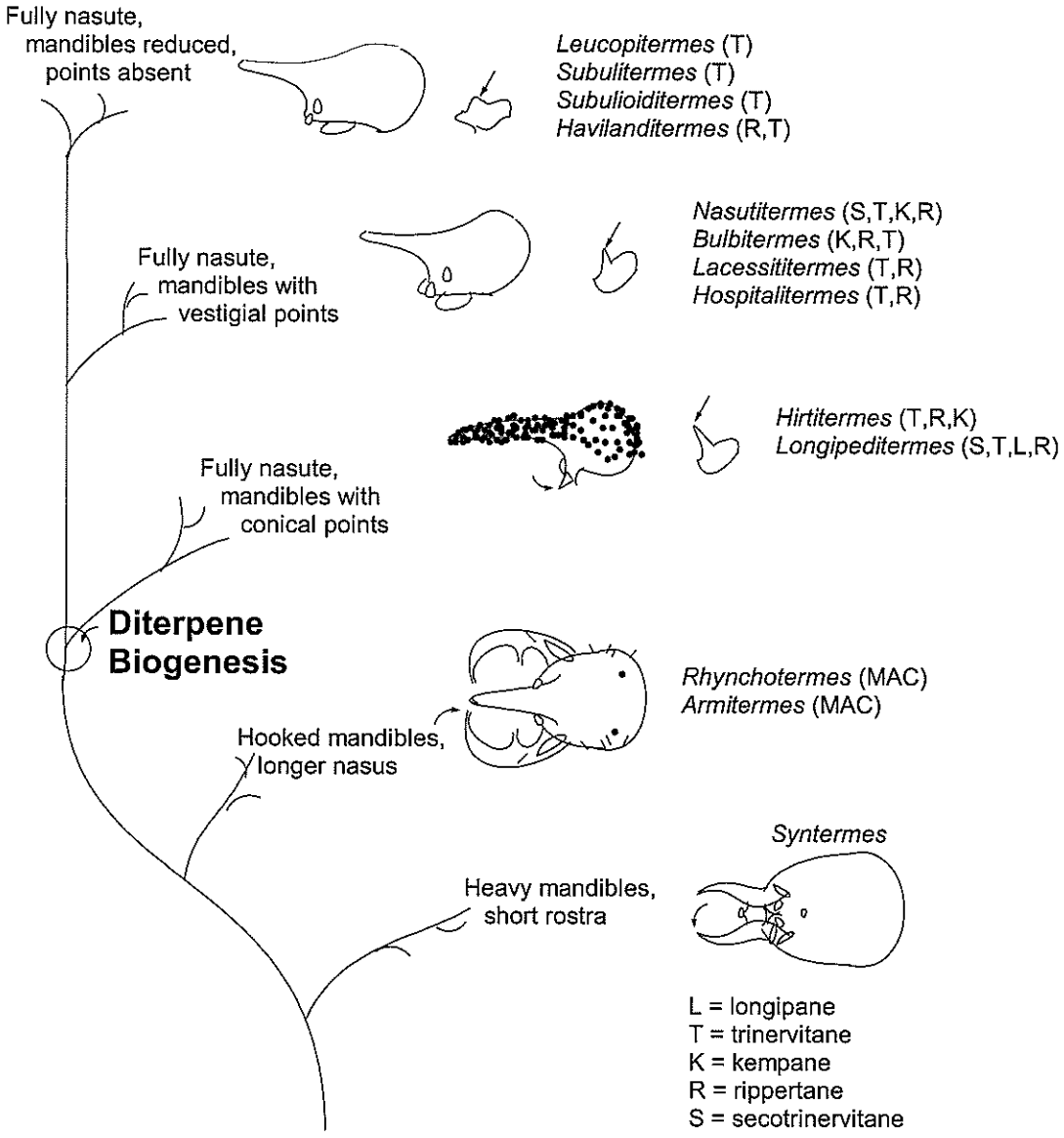

Figure 2. Monophyletic regression of soldier mandibles concurrent with evolution of chemicals.

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Value addition of agro-industrial wastes using solid state fermentation technology – problems and possibilities with special reference to Malaysia

G. Nagendra Prabhu
Department of Biotechnology, Asian Institute of Medicine, Science & Technology,
Amanjaya Campus, Sungai Petani, 08000, Kedah Darul Aman, Malaysia
(E-mail: nagendra_prabhu@aimst.edu.my)

Abstract Solid state fermentation (SSF), which deals with the controlled growth of microorganisms on the surface of water-insoluble substrates in the absence of free water, is a highly useful technique for value addition of agro-industrial and other lignocellulosic wastes. This technique has been successfully used by many countries for a variety of processes such as production of industrial enzymes and mushroom cultivation apart from protein enrichment of animal feed, solid waste disposal and biodegradation of hazardous organic compounds such as pesticides. However, the large-scale application of this technology has been hampered by many inherent drawbacks associated with it. Several approaches are being tried to eliminate these drawbacks and make SSF a feasible alternative to the conventional submerged fermentation technique (SmF). This paper briefly reviews the possibilities of using solid state fermentation technique in Malaysia for value addition of the abundantly available agro-industrial wastes. Various advantages possessed by the country, problems associated with the adoption of this technology and possible solutions are also discussed.

Keywords solid state fermentation – agro-industrial wastes – bioprocessing – value addition

INTRODUCTION

Solid State Fermentation (SSF) is the culturing of microorganisms on the surface of moist solid substrates in the absence of free water. During fermentation, the microorganisms grow and utilize the nutrients available from the substrates, by adsorbing onto the surface of the solid particles. It is a relatively simple, low cost technology with a long tradition in oriental food production, ensiling and composting [1]. It is also a globally recognized process for obtaining *"High Volume Low Value"* products such as traditional fermented foods, mushroom cultivation, protein enrichment of animal feed, single cell protein production, production of ethanol, organic acids, enzymes, food flavours, antibiotics, biopesticides and in the disposal of solid wastes [2-5]. Majority of the SSF processes utilizes materials of plant origin especially agricultural wastes, lignocellulosic materials such as wood, straw and grasses. An essential prerequisite of the potential substrates is that the microorganisms must be able to derive energy and nutrients from these materials. Moreover, they should be cheap and readily available throughout the year [6]. Traditionally, fungi are the preferred microorganism of choice for SSF, but bacteria, yeasts and actinomycetes are also used.

The technique of SSF offers several advantages over the conventional submerged fermentation technique (SmF), such as the presence of product in higher concentration, lower downstream processing costs, direct utilization of the fermented product as a source of crude target product and the utilization of the spent residue and/or byproducts as animal feed or fertilizer. The SSF technique will be all the more useful if the production is on site using locally available substrates, or in situ as part of a bioprocessing scheme. The in situ or on site SSF products can be preserved and stored in the dried form for months without losing potency, in contrast to the fermented broth produced in SmF [2, 3]. Further, SSF could be conducted under non-

sterile conditions by unskilled labourers.

This paper briefly reviews the need and suitability of employing the technique of solid state fermentation for the optimal utilization of the abundantly available lignocellulosic materials in the country. It also presents the advantages that Malaysia possesses in adopting such a technology, the problems and possible solutions as well as the priorities for action in implementing this technology.

INTERNATIONAL SCENARIO

According to Gavrilescu and Chisti [7], the biotechnology industry has already become a sustainable alternative for chemical industry. Several studies have already indicated the suitability of using the SSF technology for value addition of the agro-industrial wastes [8, 9]. Most of the Oriental countries have adopted this technique for several centuries for the production of traditional fermented foods [10]. Studies in the author's laboratories in India have reported on the success of using five locally available solid substrates, wheat bran, rice husk, copra cake, groundnut cake and saw dust, for the production of bacterial L-glutaminase enzyme under SSF [11, 12]. The common aquatic weeds, Water Hyacinth (*Eichhornia crassipes*) and Water Moss (*Salvinia molesta*) have also been recently reported as solid substrates for the production of bacterial cellulase [13, 14].

A large scale SSF plant has been set-up in Calcutta, India by *Balmer-Laurie* in collaboration with the Indian Institute of Technology, Kharagpur, to process 70 tons of wheat bran per day for protease enzyme production. Pilot plant level facilities are available at the Central Food Technological Research Institute, Mysore, India. *Biocon India*, India's largest Biotechnology company located in Bangalore, India has developed a fully automated bioreactor for SSF production of drugs such as lovastatin and also received approval from the US-FDA for the process [15]. Pilot-scale SSF plants are in operation in several other parts of the world such as Dijon, France and Georgia, USA. SSF based commercial production facilities also exist in Germany and USA [16]. In Germany, *Prophyta GmbH* has developed a unique technology for the mass production of filamentous fungi, up to 500 kg fungal biomass a day, for use in SSF. The commonly used strains for SSF in Germany are *Coniothyrium minitans, Paecilomyces lilacinus, Verticillium lecanii, Talaromyces flavus, Metarhizium anisopliae, Beauveria* spp., *Trichoderma* spp., *Gliocladium* spp., *Ophiostoma* sp., *Monascus* sp., *Sepedonium* sp., *Penicillium* spp., *Sclerotinia* spp., *Fusarium* spp., *Claviceps purpurea, Pseudocercosporella* sp. and *Pleurotus* sp. A company called Lyven was born out of a common research program between the French sugar group Saint Louis Sucre and the French Research Institute INRA based at Dijon. They are now manufacturing a range of enzymes such as bacterial & fungal a-amylase, acid and neutral cellulases, fungal pectinases and cellulases, hemicellulase, alkaline and acid proteases, glucose oxidase, ß-glucanase and fungal pentosanase-xylanase by employing SSF. A 4000 L Pilot-scale solid-state bioreactor is available in the University of Tuscia, Italy for conducting scale-up experiments on SSF processes. A USA based multi-national company called Slyvan Inc has developed SSF technology to produce a variety of commercial microbial products such as biopesticides, secondary metabolites, nutraceuticals, and specialized mushrooms. SSF technology has been successfully used in Brazil for the utilization of agro-industrial wastes especially cassava bagasse, coffee husk and pulp, sugar cane bagasse, apple pomace, soybean and potato wastes [17]. According to Holker and Lenz [18], the field of SSF is expected to be used in several areas such as production of fungal spores, secondary metabolites, enzymes, protein enrichment of fibre-rich natural materials and bioconversion. The advantages of using SSF technique in the food industry has been recently reviewed by Couto and Sanroman [19] who concluded that there are several advantages for SSF processes over the conventional SmF in the food industry.

ADVANTAGEOUS FACTORS FOR MALAYSIA

In addition to the pro-business and politically stable Government, there are several factors that offer tremendous advantages to the country for the successful implementation of SSF technology. Some of them are listed below:

- Abundance of raw materials suited for SSF.

- Mega-biodiversity, including microbial diversity.
- Availability of skilled and unskilled labour.
- Good network of road/rail, air and waterways which means that raw materials and products can be transported to and from any corner within a few hours.
- Priority status for agriculture and related biotechnology industries by the Government.
- High literacy and science awareness among the people and among farmers.
- Interest and involvement of media (TV, Radio and Newspapers) in agriculture and related areas.

This means that any technology can easily be dispersed to the common man and applied to the field in a relatively short span of time, when compared to other countries. A brief list of commonly available raw materials, potential products and their potential uses is given in Table 1, as a general guideline. It may be noted that for some of the raw materials listed, the amounts available may not be sufficient to establish commercially viable large-scale industry, but nevertheless useful at micro-level for specific purposes like cultivation of mushrooms, animal feed and soil conditioners.

Some specific areas where SSF can help include:

- Bioconversion of lignocellulosic wastes into sugars and alcohol. This is a very important area since the Government of Malaysia is slowly reducing the fuel subsidies and is promoting R & D for biofuel.
- Conversion of solid organic wastes into Organic manure and Biofertilizer. This will help in reducing the application of environmentally harmful chemical fertilizers.

PROBLEMS

No technology can be implemented without having to solve problems associated with them. Some of the important problems that can be foreseeable at this juncture are given below.

- Limited availability of existing transferable technologies.
- Lack of coordinated research in this area.
- Absence of a national microbial culture collection, where useful microorganisms can be maintained for long periods of time and made available for R & D activities and/or commercial purposes.
- Shortage of capital investment if this technology is adopted on a large-scale.
- Shortage of adequately trained labour.
- The lack of interest among the young Malaysians to venture into agriculture and related industries.

Table 1. List of raw materials, possible products and their uses.

Raw materials	Products	Uses
Prawn & Fishery wastes	Chitinase, Animal Feed	Various Industries, Animal Husbandry
Paddy straw	Enzymes, Mushroom, Animal Feed	Various Industries, Food, Animal Husbandry
Wheat & Rice Bran	Enzymes, Flavours, Organic acids, Mushroom	Various Industries
Vegetable wastes	Enzymes, Biofertilizer, Ethanol	Various Industries, Agriculture, Energy
Aquatic weeds	Enzymes, Soil conditioners, Biofertilizer, Paper pulp, Ethanol	Various Industries, Agriculture
Sugar Cane bagasse	Organic acids, Enzymes, Mushroom, Paper pulp, Ethanol	Various Industries, Food, Agriculture
Saw Dust	Enzymes, Mushroom	Various Industries
Used Tea leaves/dust	Alkaloids, Enzymes	Various Industries
Tapioca peel	Organic acids, Enzymes	Various Industries
Palm Oil Mill wastes	Enzymes, Alkaloids, Organic acids	Various Industries

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Research and innovation towards sustainable palm oil production

A. C. Soh, K. K. Kee and K. J. Goh
Advanced Agriecological Research Sdn. Bhd., Locked Bag 212, Post Office,
47000 Sg. Buloh, Selangor, Malaysia
(Email: aarsb@po.jaring.my)

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Abstract The sustainability of the palm oil industry in addressing issues involving People, Planet and Profit is being challenged. Being a commodity crop, high yield and consequent lower production cost defines its profitability. The industry is also facing stagnating low yields and stiff competition from lower cost palm oil and soybean oil producers. It is also blamed for destroying forests, endangering sensitive ecosystems and rare species, displacing indigenous people and exploiting labour. The low national yield stems from inefficient management/labour and the lower yielding palm varieties planted.

Improved varieties with very high yield potential yet easy to harvest and possess added value traits are being bred but progress has been slow. Fortunately, with the breakthroughs and developments in tissue culture propagation, genetic transformation and marker assisted selection, variety development in oil palm can be expedited. The existing gap between the yield potential of the site and the realized yield is related to agro-management deficiencies. Research progress made in more efficient use of costly fertilizers through the nutrient balance/precision agriculture approach, recycling of field organic waste and innovative use of leguminous cover crops to reduce soil and nutrient losses due to erosion and to fix and return nutrients and organic matter to the soil, promotes sustainable crop production. The main constraint lies in the inability of management/labour to efficiently implement the prescribed agronomic inputs derived from the research results. Innovative use of computer, global positioning, geographical information and remote sensing technologies combined with automation and mechanization will resolve this.

Good agricultural practices have always been the hallmark of sustainable palm oil production and will continue to improve. Loss in biodiversity currently warrants attention. Intensification and perseverance in research and innovation will ensure the future sustainability of palm oil production.

Keywords oil palm – breeding and agronomy – productivity and sustainability

INTRODUCTION

The sustainability of the palm oil industry is being challenged although the crop has gone through three to four replanting cycles. Sustainability of any industry in the current context of corporate social responsibility has to address the issues involving People and the Planet besides Profit [1].

Palm oil has been considered to be the golden crop of Malaysia. Its prices have been generally good for the past four to five decades. It has spawned a host of oleochemical industries and enabled the country to withstand two recent economic crises. It is the second largest revenue earner in the country, worth about RM30 billion annually and employs half a million people directly and two million indirectly. Nevertheless, in terms of profitability it faces a number of critical issues involving productivity which threaten its sustainability. Palm oil is essentially a commodity crop where high yield and consequent lower production cost is crucial to its profitability. The national average yields are low and have stagnated around four tons oil per hectare for the past 20 years [2]. The yield potential of its existing planting materials is not high enough and the breeding improvement appears slow. It also

SOLUTIONS, RECOMMENDATIONS AND PRIORITIES FOR ACTION

It is suggested that there should be coordinated effort in this direction, followed by implementation. The various technologies already available should be evaluated and those found feasible should be tried first in an experimental scale at selected State/District levels with the active participation of the farmers and various Governmental and Non-Governmental agencies. Financial support can be obtained from National and International agencies. Research into appropriate technologies, suited for local level needs, should be encouraged at Universities and Research Laboratories.

The following suggestions are put forward for further discussion and appropriate action.

1. Formation of a Task Force on SSF, involving experts in the concerned fields.
2. Coordination of SSF research in Malaysia - transfer of existing technology, identification of areas for further R & D - both basic and applied as well as short term and long term.
3. Inclusion of SSF in the Agriculture/ Biotechnology/Microbiology curriculum as well as Vocational Courses.
4. Conduct of short term training programmes for farmers, small & medium entrepreneurs.
5. Farmer-participatory development of technology, revalidation and technology transfer suited to local needs.
6. Inclusion of such technologies in the purview of 9th Malaysia Plan.
7. Ensuring funding from MOSTI, IRPA, UNDP, UNIDO, UNEP etc and soft-loans from Banks for research and establishment of small-scale plants.
8. Ensuring participation of State & Central Government Departments, Non Governmental Organizations and Farmer's Cooperatives.
9. Creation of public awareness and ensuring their participation.
10. Creation of a website for dissemination of information regarding this technology.

CONCLUSION

Malaysia has a fairly high density of population with sufficient number of skilled and unskilled personnel. The pressure on natural resources is very high and an optimum utilization of all the available resources and land is ideal as a long term conservation strategy. The general decline in prices of agricultural commodities has resulted in the need for augmenting farmer's resources by diversification and value addition of agricultural by products. The challenges posed by technology and globalization should be countered by encouraging enterprise, innovation and utilization of local level resources and manpower in a well planned manner. Since Agro-biotechnology and Industrial Biotechnology are two of the three thrust areas identified by the Government of Malaysia under its new Biotechnology Policy released in April 2005, this approach is all the more relevant [20, 21]. If Malaysia is to achieve developed nation status by the year 2020, the focus should shift to a resource-oriented approach.

It can be concluded that there is tremendous potential for the optimal utilization of the natural resources of Malaysia by employing suitable techniques. The country has tremendous biodiversity and abundance of resources, a well established oil palm and rubber industry with adequate human and financial resources to initiate and implement new technologies. The process of solid state fermentation promises to be such a technology, which can easily be adopted without much investment. This is not the case with other high technology areas usually associated with modern biotechnology. The incubation period will be comparatively smaller and the financial returns will be much faster. The attention of Scientists, Administrators, Planners and all concerned should immediately focus on this aspect. It is hoped that the Scientists, Government and NGOs will consider this suggestion seriously and initiate appropriate action at the earliest.

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X-ray and EUV emission characteristics of a vacuum spark

S. H. Chew and C. S. Wong
Plasma Research Laboratory, Physics Department, Science Faculty,
University of Malaya, 50603 Kuala Lumpur, Malaysia
(Email: caseycsh@perdana.um.edu.my, cswong@um.edu.my)

Abstract Extreme Ultraviolet Lithography (EUVL) which utilizes radiation in a 2 % wavelength band around 13.5 nm is the most promising candidate for realizing the production of semiconductor chips with critical dimensions of 50 nm and below. A number of EUV radiation sources are being developed in the world to comply with this demand. The vacuum spark (UMVS-III) being a compact pulsed plasma discharge has been investigated in this laboratory as a possible EUV source. An extension of the earlier research work on X-ray production by the vacuum spark to the EUV region is carried out. The vacuum spark is powered by a single 1.85 μF Maxwell capacitor and the operating pressure is at ~10^{-4} mbar. In this work, copper and tin plasmas produced in the vacuum spark are studied as sources of EUV radiation. Some results on the X-ray measurements from the vacuum spark obtained using XRD, PIN diodes and X-ray spectrometer are presented in this paper. The electron density was estimated to be in the range of 10^{20} to 10^{23} cm^{-3} and the electron temperature was from 2 to 3 keV. Also, measurement of the 13.5 nm EUV radiation has been carried out using a detector based on the principle of photoelectric effect. Further work such as time-resolved EUV spectroscopy is being carried out to characterize the EUV emission from copper and tin anodes.

Keywords EUV source – EUV lithography – vacuum spark

INTRODUCTION

A 13.5 nm EUV source is needed to match the manufacturability of the highly reflective multilayer optics that proposed in semiconductor industry [1]. At present, plasma based EUV sources such as laser produced plasmas and gas discharges are considered by researchers worldwide as the practical light sources for Next Generation Lithography (NGL) [1-4]. These EUV sources offer several advantages over the complex and high cost ownership synchrotron sources. Various working elements including tin, lithium, xenon, oxygen, beryllium and silicon have been investigated for the generation of EUV radiation in the 13.5 nm wavelength region [2-8]. The vacuum spark is one of the well known pulsed plasma devices capable of producing hot, dense and highly ionized plasma that emits intense X-ray from solid targets [6, 9-14]. Recently, the vacuum spark has attracted new research interest as it is regarded as a potential EUV source for NGL. Vacuum sparks seem to offer an alternative with much higher conversion efficiency in tin and lithium plasmas [2, 8]. By using suitable anode materials, the vacuum spark will allow the generation of photon emission in the EUV spectral range. Thus, generation of EUV radiation from vacuum spark can be a very attractive alternative. In this work, two possible anode materials, Cu and Sn, have been investigated in a 370 J vacuum spark, which exhibit strong emission in the spectral range of interest.

EXPERIMENTAL SET-UP

The setup of UMVS-III used for generating X-ray and EUV radiations is schematically illustrated in Figure 1. The vacuum spark employs a single 1.85 μF Maxwell capacitor. The whole system is continuously pumped down by using a diffusion pump backed by a rotary pump. The system consists of the cathode and anode mounted on holders, which are placed in a vacuum chamber. The cathode is a

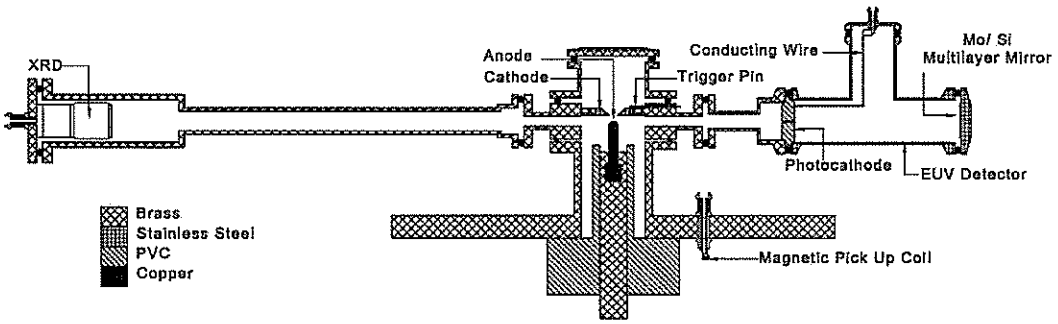

Figure 1. The schematic diagram of the vacuum spark system. The PIN diode is not shown in the diagram because it is mounted at the port perpendicular to the view.

stainless steel disk with an aperture (5.0 ± 0.3 mm) along the axis and tapered towards the replaceable anode. The electrons released from the triggering pin are accelerated in the high electric field favored by the transient hollow cathode effect towards the anode. The electrons bombard at the anode will vaporize some of the anode materials which is heated by the discharge to form a plasma that generates light emission in the UV, EUV, soft X-ray and hard X-ray region. A magnetic probe is implemented to monitor the rate of change of current. A Quantrad model 100-PIN-250 diode, a PBX 65 PIN diode and an X-ray diode (XRD) are used as X-ray detectors to measure the X-ray emission. In addition, an X-ray spectrometer (XR-100CR) is used to determine the X-ray spectrum. To monitor the EUV emission at wavelength of 13.5 nm, a detector based on the principle of photoelectric effect is employed.

RESULTS AND DISCUSSION

The X-ray production from UMVS-III had been studied over the years. The discharge was triggered by the electron beam generated by the transient hollow cathode effect [12] and operated with pressure of ~1 $\times 10^{-4}$ mbar. Previous experiments [9, 11-13] using this device with copper anode (Z=29) gave an estimated electron temperature of 2 to 3 keV.

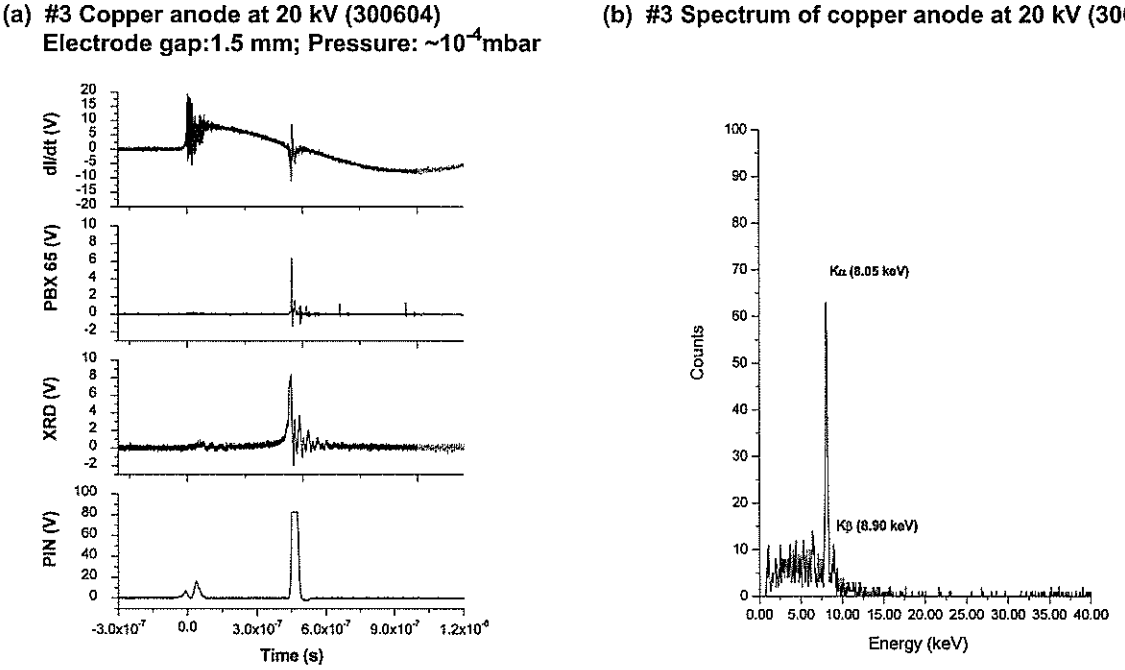

Figure 2. (a) dI/dt and X-ray signals at 20 kV for copper anode and (b) the corresponding X-ray emission spectrum. In (a), the side-on PIN diode signal is off-scale resulting in a flat top pulse.

The electron density deduced was in the range of 10^{20} to 10^{23} cm^{-3}. X-ray emitted from the hot dense plasma spot was observed consistently. From the pinhole photography, the size of this hot spot formed was measured to be of the order of 100 to 200 μm [9, 12, 14, 15]. The total emission power and total emission energy of the hot spot were calculated to be 4.0×10^5 -6.0×10^5 W and 3.0×10^{-3} -6.0×10^{-3} J [9]. It was found that the X-ray emission spectrum was dominated by the continuum radiation and the characteristic K_α lines of the material. The continuum radiation and K_α lines are observed from both the interaction between the pre-breakdown electron beam and the anode and the plasma emission. Also, it was noted that the optimum discharge voltage for producing efficient X-ray radiation was at 20 kV [11]. Figure 2 shows the typical results of the X-ray measurements obtained for a 20 kV copper discharge and at operating pressure of ~10^{-4} mbar.

The preliminary results of our investigations of vacuum spark plasma both with copper and tin anodes as EUV sources are presented as well. The working pressure throughout the experiment is ~10^{-4} mbar. In this case, the discharge voltage is scaled down to 5-14 kV since the emission in the EUV region is predominantly emitted at this voltage range. An EUV detector based on the fundamental principle of photoelectric effect is used to characterize the EUV emission from the plasma. The EUV detector [16] uses a Mo/Si multilayer coating mirror to selectively reflect the EUV radiation at the wavelength of 13.5 nm. The Mo/Si multilayer reflective mirror has a peak reflectivity near 65 % at the wavelength of 13.5 nm when the angle of incidence is 5 degrees off normal (Fig. 3). A photocathode is used to detect the EUV photons that reflected from the mirror and the corresponding photocurrent generated is recorded by an oscilloscope. Thus by utilizing a Mo/Si multilayer coating mirror in the design of the EUV detector, the time evolution of EUV emission from the vacuum spark plasma can be observed.

The population density distributions for copper and tin plasmas are computed assuming the Coronal Equilibrium (CE) model (see Fig. 4) [H.S. Poh, pers. comm.]. For copper plasma, emission lines at wavelengths around 13.5 nm may be expected from Cu^{9+}, Cu^{10+} and Cu^{11+} species. Cu^{11+} specie is dominant at an electron temperature around 46 eV. At this electron temperature, the continuum emission also peaks at around 13.5 nm. Tin (Z=50) is more advantages compared to copper since it is shown to have many excited states from Sn^{10+} - Sn^{14+} ions at wavelength of near 13.5 nm. These prominent species are predicted for electron temperatures from approximately 30-60 eV.

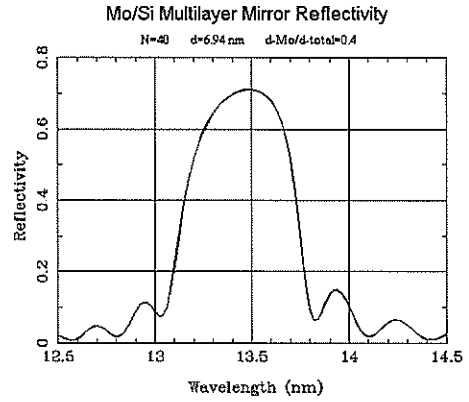

Figure 3. Near normal incidence reflectivity of a Mo/Si multilayer mirror as a function of wavelength.

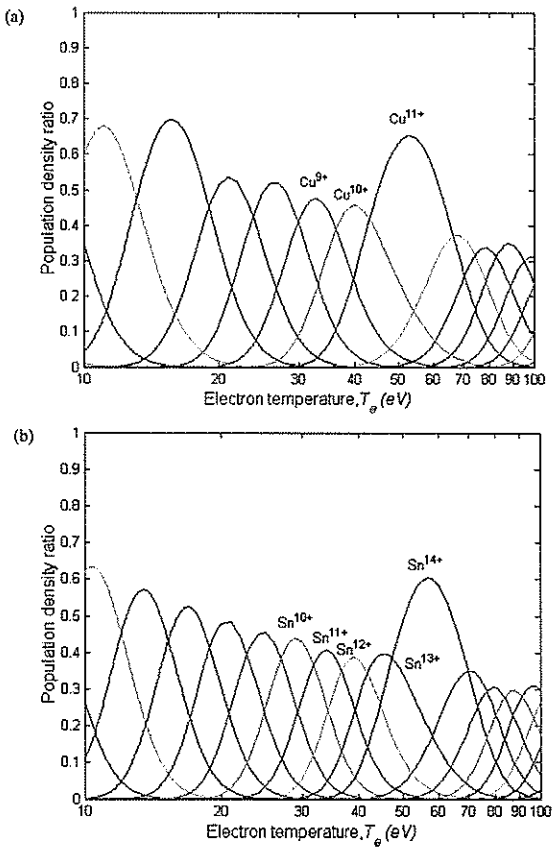

Figure 4. The population density ratio of (a) copper and (b) tin ionic species versus electron temperature calculated from the Coronal Equilibrium Model.

Figure 5 demonstrates a copper vacuum spark discharge at 10 kV and electrode spacing of 1.5 mm. In this discharge, the emissions from the plasma are recorded simultaneously with the rate of change of discharge current. Both the soft X-ray pulse in the XRD and EUV signal in the EUV detector are observed to follow the dI/dt signal. It can be seen that the EUV and X-ray photons are emitted during the rise and the drop of dI/dt signal. The light intensities from the EUV detector and XRD reach their peaks about 650 ns after the breakdown of the vacuum spark and then decay gradually indicating the decay of the plasma. It is observed that the EUV signal has a duration of about 1.5 μs. The EUV emission with the highest intensity is obtained after the peak of the dI/dt signal. It is clear that strong peaks from the EUV detector and XRD signals coincide with the dI/dt dips. Furthermore, the EUV emission signal is observed to correspond well with the XRD signal. It can also be seen that there is a hard X-ray pulse detected from the PIN in this case.

A tin vacuum spark discharge at 10 kV with electrode spacing of 1.9 mm is shown in Figure 6. In this discharge, no dip is being observed in the dI/dt signal. Also, no significant peaks are observed from the XRD and EUV signals in the detectors. In addition, it can be seen that there is no hard X-ray pulse detected from the PIN diode. This indicates that the plasma has not been heated enough to give emission in the hard X-ray region. However, the EUV detector registers a broad pulse of duration about 1.5 μs. The pulse shapes of different discharge voltages are found to be identical in the EUV signals. Generally, significant EUV emissions are observed for discharges with discharge voltage of 5 kV to 14 kV in this series of experiments. It is noted that both EUV and XRD signals are enhanced as the charging voltage is increased.

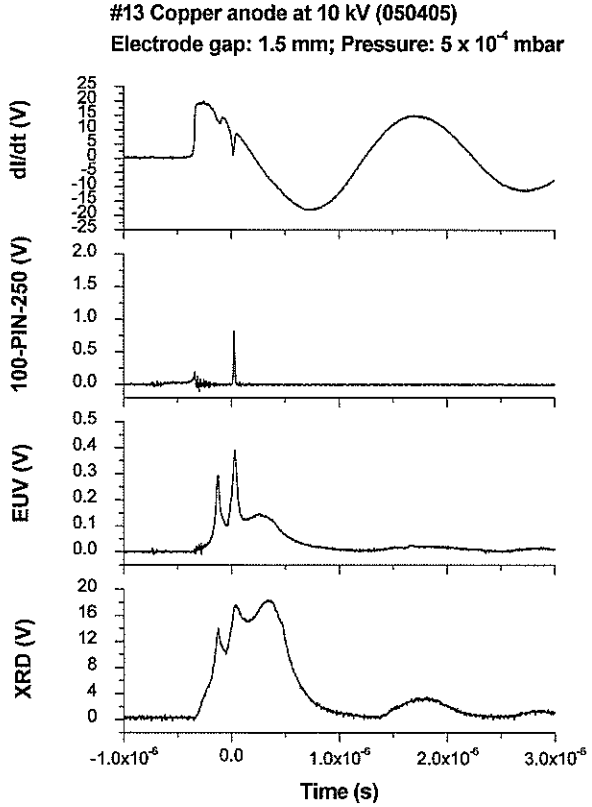

Figure 5. dI/dt, EUV signal and X-ray intensity at 10 kV for copper anode.

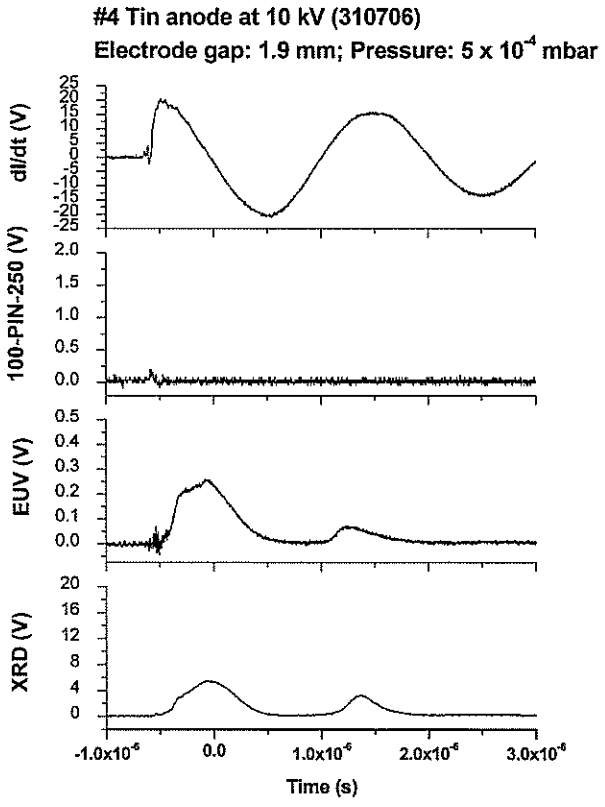

Figure 6. dI/dt, EUV signal and X-ray intensity at 10 kV for tin anode.

CONCLUSION

Measurements of EUV emission suggest that besides being an intense X-ray source, the vacuum spark may also be utilized as a pulsed EUV source for nanolithography. Further work is being carried out to characterize this EUV source.

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Bound-state energies of a particle within a finite rectangular potential well

Bernardine Renaldo Wong
Quantum Scattering Theory Group, Institute of Mathematical Sciences, Faculty of Science, University of Malaya,50603 Kuala Lumpur, Malaysia
(Email: bernardr@um.edu.my)

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Abstract We present a simple method leading to a formula for calculating the energies of a bound state of a particle confined within a finite rectangular potential well. This formula permits us to know the maximum number of bound states supported by the system. Elementary iterations of the formula yield rapidly-converging eigen-energies.

Keywords time-independent Schrödinger equation – finite rectangular potential well

INTRODUCTION

The time-independent Schrödinger equation (TISE) is a fundamental equation in non-relativistic quantum mechanics. Analytical solutions for the bound-states of the TISE are available for only a few families of potentials, such as the infinite rectangular well and the harmonic oscillator [1]. Perhaps the simplest potential for which the TISE needs to be solved numerically is the finite rectangular well potential. The rectangular potential well is defined as

$$V(x) = \begin{cases} V_0 & \text{for } |x| > a \\ 0 & \text{for } |x| \le a \end{cases} \quad (1)$$

where $V_0 > 0$ is the well depth, and $2a$ is its width. In the usual approach to solve for bound states eigen-energies, the wave function is expressed as

$$\psi(x) = \begin{cases} A\exp(\kappa x) & for\ x < -a \\ B\exp(ikx) + C\exp(-ikx) & for\ -a \le x \le a \\ D\exp(-\kappa x) & for\ x > a \end{cases} \quad (2)$$

where the wave number $k=\sqrt{2mE/\hbar^2}$ and $\kappa=\sqrt{2m(V_0-E)/\hbar^2}$, while m is the particle mass and $\hbar$ is the reduced Planck constant. E is the energy measured with respect to the base of the well. The continuity of the wave-function and its spatial first-derivative is invoked at the boundary points $x = \pm a$ in order to obtain a system of four linear equations involving the matching coefficients A, B, C and D. Solutions of this system exist only for certain wave numbers k, which in turn fix the eigen-energies E. The conventional approach is to separately treat the even- and odd-parity solutions [2]. Recently, Bloch and Ignatovich [3] presented an alternative method of determining the eigen-energies which makes use of self-consistent reflections of the wave function off the walls.

SELF-CONSISTENT REFLECTION METHOD

Following Bloch and Ignatovich [3], consider the propagation amplitude of a particle $e^{ik(x+a)}$ from left to right in the domain $-a < x < a$. This is multiplied by the probability amplitude for reflection at the right boundary, $\rho=(k-i\kappa)/(k+i\kappa)$. Following this, the reflected wave subsequently propagates to the left with amplitude, $e^{-ik(x-a)}$. There is another reflection at the left boundary, after which the particle returns to its initial position. However, since the bound-state is a stationary state, we obtain the quantization condition

faces stiff competition from lower cost palm oil and genetically modified (GM) soybean oil producers (e.g. Indonesia, Brazil, Argentina).

The industry is also being blamed for destroying forests, endangering sensitive ecosystems and rare species, displacing indigenous people and exploiting labour.

EFFORTS IN ADDRESSING THESE ISSUES

Genetic improvement approach

Dwarf Variety. The main reason behind the low stagnating national yields is the existence of tall old palms which still constitute a significant portion (ca. 20%) of the existing national plantings. Tall palms are difficult to harvest resulting in lower yield. Replanting with younger palms has also been slow (ca. 3%) due to high initial development cost cum revenue lost during the immature period. High yielding oil palm varieties planted also tended to grow tall fast. Dwarf varieties are the obvious solution. Breeders who discovered the Dumpy dwarf mutant in the 1950s foresaw its advantage in facilitating harvesting and prolonging the crop's economic life [3]. However, in the quest for high early yields that resulted in fast growing varieties, this was largely forgotten. AAR (Advanced Agriecological Research), however, pursued the idea of a dwarf high yielding variety, by incorporating the Dumpy dwarf trait into the tall high oil yielding AVROS variety by backcross breeding (Table 1; Figs. 1,2). This Dy.AVROS semi-dwarf variety has been represented in about 15-20% of the annual plantings since the early 1980s. The benefit of the slower growing habit would translate to about RM20 billion with the extended four years of harvesting or crop life [4,5].

While dwarf palm varieties improve recoverable yield, yield still needs to be improved further because of faster yield improvement in competing oil crops e.g. GM soybean, and lower cost palm oil producers e.g. Indonesia.

High Yield Potential Variety. To improve the genetic yield potential of a crop, the ideotype breeding approach is needed [6]. Taking the cue from such success in cereal crops e.g 'miracle rice' [7], an oil palm ideotype is one with a short thin trunk, short leaves to capture more light, is tolerant of higher density planting and consequently of high biomass production, and produces many smaller bunches with high oil content [8,9]. Such high Harvest Index (HI) palms put more of the energy captured into yield than to vegetative growth. The combination of high biomass production and high HI traits results in higher yield potential. The Dy.AVROS genotype is still deficient in a number of desirable ideotype traits in having a thicker trunk, heavier canopy, bigger but fewer bunches with moderately high oil content. The Ybi.AVROS genotype possesses many of these desirable traits except for its height. By crossing the Dy.AVROS with the Ybi.AVROS and selecting from the transgressive segregants, the

Table 1. Comparisons between Dumpy.AVROS (Dy.AVR) and AVROS (AVR) DxP hybrids in oil yield and palm height growth.

Trait	Hybrid	AAR Trial 1	AAR Trial 2	UP Trial	Pamol Trial
Oil Yield	AVR	6.8	6.0	8.1	5.7
(t/ha/year)	Dy.AVR	6.2	6.4	7.9	4.8
$LSD_{.05}$		0.6	1.0	0.7	0.6
Palm Height	AVR	189	190	217	290
(cm)	Dy.AVR	145	167	197	210
$LSD_{.05}$		18	20	-	30
Height Increment	AVR	62	46	-	74
(cm/year)	Dy.AVR	49	40	-	58
$LSD_{.05}$		12	5	-	7

AAR Trials – oil yields averaged over first 71/2 years of harvesting, height at year 4, height increment from year 3-4 (AART1) and year 3-5 (AART2).
UP Trial - oil yields averaged over first 4 years of harvesting [5].
Pamol Trial – oil yields averaged over first 5 years of harvesting [5].

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$$\rho^2 \exp(4ika)=1. \qquad (3)$$

Equation (3) can only be satisfied for a finite number of wave numbers k_n corresponding to definite eigen-energies E_n. In order to solve equation (3), let us define the following dimensionless quantities:

$$\gamma = \sqrt{\frac{2ma^2V_0}{\hbar^2}} \qquad (4a)$$

and

$$\tau = \sqrt{\frac{E}{V_0}}. \qquad (4b)$$

Bound states can only be found in the domain $0 \leq \tau \leq 1$. Substituting equations (4a) and (4b) into (3) and performing a little algebra, one obtains

$$\exp[4i(\gamma\tau + \theta)] = 1 = \exp(2\pi in), \quad (5)$$

where n is a positive integer and $\cos 2\theta = 1-2\tau^2$. Thus, we obtain the transcendental equation for the eigen-energies as

$$2\gamma\tau + \cos^{-1}(1-2\tau^2) = n\pi. \quad (6)$$

From equation (6), it is possible to easily determine the maximum number of bound-states supported by the finite rectangular well, n_{max}. Setting $\tau = 1$, we obtain

$$n_{max} = \lfloor 1+(2\gamma/\pi) \rfloor, \quad (7)$$

where $\lfloor x \rfloor$ is the floor function, yielding the greatest integer larger than x. From equation (7), it is clear that the finite rectangular well can support at least one bound state. Furthermore, the n^{th} bound state can be found in the interval $(n-1)\frac{\pi}{2\gamma} \leq \tau_n \leq n\frac{\pi}{2\gamma}$, where $1 \leq n \leq n_{max}$. Numerical solutions for τ_n may be found by recasting equation (6) into the form $\sin(2\gamma\tau + 2\theta)=0$. An improved estimate $\tau_n^{(i+1)}$ can be obtained from the previous estimate $\tau_n^{(i)}$ using the well-known Newton-Raphson formula,

$$\tau_n^{(i+1)} = \tau_n^{(i)} - \frac{\sqrt{1-(\tau_n^{(i)})^2}\,\tan\left[2\gamma\tau_n^{(i)} + 2\theta\right]}{2\left[\gamma\sqrt{1-(\tau_n^{(i)})^2} + 1\right]}. \quad (8)$$

A good initial estimate may be found by making the approximation $\cos^{-1}(1-2\tau^2) \approx \frac{\pi}{3}\tau(1+2\tau)$ in equation (6). The resulting quadratic equation yields an initial estimate

$$\tau_n^{(0)} = \frac{6n\pi}{6\gamma+\pi}\,\frac{1}{1+\sqrt{1+\frac{24n\pi^2}{(6\gamma+\pi)^2}}}. \quad (9)$$

In the limit $\gamma \to \infty$, equation (9) results in the correct eigen-energy formula for the infinite rectangular potential well, $E_n = n^2\pi^2\hbar^2/(8ma^2)$.

Since the Newton-Raphson formula converges quadratically, few iterations of equation (8) are required. The eigen-energies E_n can then be obtained from equation (4b).

RESULTS AND DISCUSSION

As a representative case, we consider an electron

Table 1. The iteration of τ_n for the four bound states, n = 1, 2, 3, and 4. The iterations follow equation (8) with the initial estimate (i = 0) calculated using equation (9). The eigen-energies are displayed in the final row.

i \ n	1	2	3	4
0	0.24148563756521	0.46611506848388	0.67699149933686	0.87636879892252
1	0.23310721584308	0.46417254349585	0.68993974718389	0.90268333032608
2	0.23314142256222	0.46417281839328	0.68977721170279	0.90099835459829
3	0.23314142253784	0.46417281839328	0.68977720861356	0.90099678444133
4	0.23314142253784	0.46417281839328	0.68977720861356	0.90099678443963
5	0.23314142253784	0.46417281839328	0.68977720861356	0.90099678443963
E_n (eV)	0.271774614514828	1.077282026675784	2.378962987613569	4.058976027852800

bound within a rectangular well of depth V_0=10 eV and with a width $2a$ = 1.0 nm. We use $\hbar^2/me$ = 7.619×10^{-2} eV.nm^2. In this case, $\gamma = 5.728237519465$ and the number of bound states is n_{max}=4. Table 1 shows τ_n for each iteration, i, of equation (8) using the initial estimate of equation (9). The final row presents the eigen-energies, in eV, calculated using equation (4b).

From Table 1, we see that equation (9) gives a very good initial estimate being correct to within 3.5% for the bound states considered. In general, convergence is most rapid for the more tightly bound states. Typically, two iterations are sufficient for the n = 1 and n = 2 states. The weakly bound states generally require more iterations. In any case, just 4 iterations are sufficient for the state n = 4.

CONCLUSION

We have developed a transcendental formula by which it is possible to know, in advance, the number of bound states supported by a finite rectangular potential well. This formula can be used to derive a good approximation for the bound state eigen-energies. This approximation can also be used in a rapidly-convergent Newton-Raphson formula.

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Evaluation of nutrient contents and amino acid profiling of various types of palm kernel cake (PKC)

A. M. Marini[1], A. M. Yatim[2], A. S. Babji[2], B. O. Annuar[3] and S. Noraini[1]
[1]Strategic Livestock Research Centre, Malaysian Agriculture Research and Development Institute (MARDI), P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
[2]School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia
[3]School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia
(Email: marini@mardi.my)

Abstract Palm kernel cake (PKC) is a by-product from the extraction of palm oil. It is suitable for feeding beef and dairy animals. Current research emphasizes on the use of PKC for poultry feed. An attempt was made to increase the nutritional composition of PKC by fermentation with *Aspergillus niger* using solid substrate fermentation (SSF) technique. Analyses were conducted to determine the nutritional composition and amino acid profiling of fermented PKC (fPKC) compared to untreated PKC, soy-bean meal (SBM), polypeptide PKC for food (pPKC) and feed grade [pPKC(f)]. Fermented PKC was produced by SSF at laboratory scale using a tray system. The crude protein of *A. niger* fPKC (24.7 %) increased significantly compared to the value of untreated PKC (17.5 %). The fPKC contained 15.7 % of total amino acid, accounted for 63.4 % of the crude protein. Neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose and cellulose levels in fPKC were significantly decreased from 79.0 % to 50.3 %, 46.8 % to 35.8 %, 32.2 % to 14.5 % and 34.4 % to 21.5 %, respectively. The total ash content was significantly higher in fPKC (5.8 %) compared to the value in untreated PKC (4.1 %). All minerals and trace elements analyzed in the fPKC were significantly higher compared to the level in untreated PKC, except for copper and phosphorus. Results from the study showed that SSF technique could be used to increase the nutritional composition of PKC. Amongst the pPKC, pPKC(f) and SBM, the protein value of fPKC was significantly lower. The total amino acid in pPKC (f) accounted for 88.7 % of crude protein compared to the level in pPKC, which accounted for 81.4 % of crude protein. Both fPKC and pPKC (f) could be considered as potential alternative energy and protein sources for monogastric animals.

Keywords fermented palm kernel cake – polypeptide palm kernel cake – nutritional value – amino acid profile

INTRODUCTION

Palm kernel cake (PKC) (by-product of palm oil milling) is abundantly produced throughout the year in Malaysia and this guarantees their supply and availability as a major ingredient for livestock feeding. The PKC comprises mainly of cell wall which consists largely of polysaccharides such as mannan which is responsible for the low digestibility of PKC by monogastric animals [1]. Mechanical and chemical processes could increase the digestibility of feedstuffs without altering its nutrient content significantly. Through biological processes such as fermentation, it is possible to improve digestibility, protein efficiency ratio and amino acid availability of the PKC. During fermentation, improvements in vitamin content and destruction of anti-nutritional factors could also be achieved due to the presence of related enzymes with the substrate [2].

Solid substrate fermentation (SSF) is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid [3]. Research on the selection of suitable substrate

for SSF has mainly been centred on agro-industrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest part of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium [4]. Apart from this, fungal growth under solid state conditions has also been found to be more suitable for low technology applications, and there is hardly any waste disposal at the end because the whole product may be used directly in animal feeds [5]. In addition, costs are much lower due to the efficient utilization and value-addition of wastes [6].

The process of utilizing enzymes from microbes in the biotechnological fermentation of PKC has been studied extensively by Malaysian Agriculture Research and Development Institute (MARDI) [7]. It was found that the fibre component could be degraded resulting in higher energy value and better improvement on animal performance. Based on a report by Daud et al. [7], the metabolisable energy (ME) of *A. niger* fermented PKC increased from 6.2 MJ/kg to 9.0 MJ/kg. The on-going R&D effort in MARDI is an up-scaling production of improved PKC, increasing the metabolisable energy to 9.5 MJ/kg and an inclusion of 50 % in the poultry diet.

The objectives of the present study were to determine the amino acid profiles, chemical properties and nutritional value of *A. niger* fermented PKC compared to untreated PKC, soy-bean meal (SBM), polypeptide PKC for food (pPKC) and feed grade [pPKC(f)].

MATERIAL AND METHODS

Solid substrate fermentation (SSF) of PKC

In this study, raw material "expeller pressed palm kernel cake" was bought from FELDA Factory in Serting. While, established fermentation technique by PKC group in MARDI for the production of improved PKC using SSF technique was employed in this study [8]. The sterile PKC substrate (1.3 kg in tray system) with 50 % moisture content was inoculated with 0.5 % of *Aspergillus niger* (FTCC 5003) spore suspension (x 10^7 spore/ml). The culture was incubated at 30 ^{0}C for 66 hours. The fPKC was then dried in an oven for 48 hours at 60 ^{0}C. Samples from different batches of fermentation were then ground, mixed and sieved to get smaller particles (1 or less than 1 mm) and homogenized.

Polypeptide PKC

Polypeptide PKC is a crude protein of PKC. It is obtained by an enzyme extraction. Two types of pPKC were received from Wuhan Tallyho Institute of Polypeptide, Wuhan, China. The higher grade of pPKC is used for human consumption (food grade), while the lower grade is for animal feed [pPKC(f)]. Protein qualities of these two materials were compared with SBM.

Soy-bean meal

Soy-bean meal (45 % crude protein) was bought from QL Feed Mill in Port Klang. In this study, SBM was used as a standard of protein source in animal feed.

Proximate and mineral analyses

Proximate analyses (crude protein, moisture content, ash and crude fat) of samples were determined according to standard methods described by the Association of Official Analytical Chemists [9]. All nitrogen was determined using the micro-Kjeldahl procedure [9]. The protein content was calculated by multiplying the total nitrogen content by a conversion factor of 6.25. Dry matter is the difference between the original weight of the sample and the weights of its water. While, nitrogen–free extract is the difference between the original weight of the sample and the sum of the weights of its water, ether extract, crude protein, crude fiber and ash. Fibre analyses were carried out according to the method described by Van Soest [10]. Neutral detergent fibre (NDF) represents total fibre in plant cell walls and consists of lignin, cellulose and hemicellulose, while acid detergent fibre (ADF) represents the less digestible components which consist of lignin and cellulose. The concentration of hemicellulose was calculated as the difference between NDF and ADF, while that of cellulose as the difference between ADF and acid detergent lignin (ADL).

Mineral concentrations were determined using a flame method of atomic absorption spectrophotometer GBC HG 904 AA (GBC Scientific Equipment Pty Ltd, Australia) except for the phosphorus that was determined using adsorption method with UV-VIS spectrophotometer (TU-1800

SPC, China). Gross energy of fPKC and untreated PKC were determined by bomb calorimeter (IKA Calorimeter system, C 4000).

The free amino acids were estimated by HPLC (Waters Inc.), subjected to pre-column derivatization and determined using AccQ Taq method as described by the manufacturer (Waters Inc.). Samples were hydrolysed with hydrochloric acid, in the absence of air, to break the peptide bonds of a protein. This procedure gives good results for the acid-stable amino acids, i.e. all those commonly occurring in food proteins except cystine, cysteine, methionine, and tryptophan, which are labile under acid hydrolysis conditions and require separate method of analysis.

The amino acid analyzer detected 15 amino acids, as during acid hydrolysis asparagine was converted into aspartic acid and glutamine into glutamic acid. The amino acids cystine, cysteine and methionine are therefore first oxidized with performic acid, under controlled conditions, to convert into their residues of cysteic acid and methionine sulphone. These acid-stable residues are then freed from the protein by hydrolysis with 6 N HCl [11,12]. While for tryptophan, it was released using an alkaline hydrolysis [13].

Statistical analyses

All statistical computations were performed with the ANOVA procedure followed by Duncan New Multiple Range Test (DMRT) of the Statistical Analysis Systems Software [14].

RESULTS AND DISCUSSION

Proximate and chemical analyses

Data on proximate analyses which were calculated on dry matter basis are shown in Table 1. The data indicated that the crude protein of fPKC (24.7 %) is significantly higher compared to the value in untreated PKC (17.5 %). The increase in the protein content of the fPKC could be attributed to the reduction of biomass after fermentation, which results from the utilization of the carbon content by the fungi. According to Mathot *et al.* [15], about 20.0 % of the initial substrate (DM) was lost during fermentation, especially starch and hemicellulose which were the main sources of carbon for the fungus. Apart from this, the increase in the amount of the microbial biomass in the form of single-cell proteins may possibly account for the increase in the protein content of the *A. niger* products [16]. Results in Table 1 also showed that pPKC(f) and pPKC contained high crude protein with 42.4 % and 45.9 %, respectively. However, these values were significantly lower compared to the value of crude protein in SBM (49.8 %) as a main protein source in poultry feed.

The total ash content was significantly higher in fPKC (5.8 %) compared to the value in untreated PKC (4.1 %) (Table 1). The difference in the total ash content might be due to the differences in the mineral concentration of the samples. The significantly decrease of carbohydrate (NFE) in fPKC compared to untreated PKC (Table 2) could

Table 1. Proximate analyses of various types of PKC and SBM (dry matter basis).

Component	PKC	fPKC	pPKC (f)	pPKC	SBM
Crude protein (%)	17.5 ± 0.3^{e}	24.7 ± 0.2^{d}	42.4 ± 0.6^{c}	45.9 ± 0.2^{b}	49.8 ± 0.5^{a}
Ash (%)	4.1 ± 0.0^{e}	5.8 ± 0.0^{d}	16.1 ± 0.1^{b}	17.5 ± 0.1^{a}	7.1 ± 0.0^{c}
Crude fat (%)	10.7 ± 0.1^{a}	4.1 ± 0.4^{b}	0.1 ± 0.0^{d}	0.2 ± 0.0^{d}	2.0 ± 0.1^{c}
Moisture content (%)	5.2 ± 0.0^{e}	8.8 ± 0.1^{c}	6.2 ± 0.1^{d}	9.2 ± 0.1^{b}	10.1 ± 0.0^{a}
Dry matter (%)	94.8 ± 0.0^{a}	91.2 ± 0.0^{c}	93.8 ± 0.1^{b}	90.8 ± 0.1^{d}	89.9 ± 0.0^{e}

Data are presented in the mean values ± sd (n = 3). Values with different letters within same row are significantly different (p <0.05). PKC = palm kernel cake; fPKC = fermented palm kernel cake; pPKC (f) = polypeptide PKC (feed grade); pPKC = polypeptide PKC (food grade); SBM = soy bean meal

be attributed to the possible transformation of some of the carbohydrate, which the organism possibly uses as its carbon source to some other metabolites, such as protein or fat [17]. However, the crude fat content in untreated PKC decreased significantly (p <0.05) from 10.7 % to 4.1 % in the fPKC (Table 1). The result tallies with the decrease of gross energy in the same material compared to the values in the untreated PKC (Table 2). Results also showed that ash content in both pPKC (f) and pPKC were significantly higher compared to the value in SBM. On the other hand, the crude fat and moisture content of these two materials were significantly lower compared to values in the SBM. These results gave an advantage for pPKC(f) and pPKC as they have a longer shelf life.

Results on fibre analyses (Table 2) also showed an improvement in nutritive value of fPKC compared to untreated PKC except for the acid detergent lignin (ADL) value. NDF and ADF levels in fPKC were significantly decreased from 79.0 % to 50.3 % and 46.8 % to 35.8 %, respectively. The significant reduction in hemicellulose from 32.2 % to 14.5 % and cellulose (34.4 % to 21.5 %) might be attributed to fibre degradation by fungal enzymes. On the other hand, the increase in the ADL content of the fPKC could be attributed to the reduction of biomass after fermentation. These findings were similar to those reported by other researchers [2,5,15] regarding utilizing *A. niger* in improving nutritional value of barley, cassava and PKC, respectively. SSF has also been reported to improve the nutritive value of food legumes and cereals by decreasing the levels of antinutrients [18,19] and increasing protein digestibility [20,21].

Table 2. Fiber, NFE and gross energy of PKC and fPKC (dry matter basis).

Chemical composition	PKC	fPKC
Crude fiber (%)	16.8 ± 0.7[a]	14.5 ± 0.3[b]
NDF (%)	79.0 ± 0.3[a]	50.3 ± 0.2[b]
ADF (%)	46.8 ± 0.2[a]	35.8 ± 0.1[b]
ADL (%)	12.4 ± 0.3[b]	14.3 ± 1.1[a]
NFE (%)	45.9 ± 0.9[a]	42.2 ± 0.6[b]
Hemicellulose (%)	32.2 ± 0.3[a]	14.5 ± 0.3[b]
Cellulose (%)	34.4 ± 0.3[a]	21.5 ± 1.1[b]
Gross energy (kcal/kg)	4884 ± 27[a]	4759 ± 39[b]

Data are presented in the mean values ± sd (n = 3). Values with different letters within same row are significantly different (p <0.05). PKC = palm kernel cake; fPKC = fermented palm kernel cake; NFE = nitrogen free extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin

Minerals and trace elements

All minerals and trace elements analyzed in the fPKC were significantly higher compared to the level in untreated PKC, except for copper and phosphorus which were just slightly higher in the fPKC (Table 3).

Table 3. Mineral and trace elements in PKC and fPKC (dry matter basis).

Element	PKC	fPKC
Ferum (ppm)	626.2 ± 48.4[b]	840.0 ± 19.2[a]
Zinc (ppm)	46.1 ± 1.9[b]	68.4 ± 2.8[a]
Copper (ppm)	23.0 ± 0.4[a]	23.9 ± 0.4[a]
Manganese (ppm)	289.8 ± 48.6[b]	415.8 ± 3.2[a]
Calcium (%)	0.3 ± 0.0[b]	0.5 ± 0.1[a]
Magnesium (%)	0.2 ± 0.0[b]	0.3 ± 0.0[a]
Phosphorus (%)	0.6 ± 0.0[a]	0.8 ± 0.1[a]

Data are presented in the mean values ± sd (n = 3). Values with different letters within same row are significantly different (p <0.05). PKC = palm kernel cake; fPKC = fermented palm kernel cake

Amino acid profiling

Results in Table 4 showed the amino acid profiles of various types of PKC and SBM. Compared with untreated PKC, the amino acid profile of fPKC was better. However, the increase of total amino acid analyzed from 14.9 % to 15.7 % were insignificant (p >0.05). The total amino acid content in fPKC accounted for only 63.4 % of crude protein, lower than the value in untreated PKC (84.9 %). The difference between total amino acid and crude protein could account for the presence of non-protein nitrogen (NPN) like nucleic acid and chitin (in the fungal mycelia) [15]. Although the total essential amino acid (EAA) in fPKC was slightly higher than in untreated PKC, the improvement in EAA indicated their higher nutritional values. The difference in total amino acid and other chemical

Table 4. Amino acid profiles of various types of PKC (dry matter basis).

Amino acid (%)	Sample				
	PKC	fPKC	pPKC (f)	pPKC	SBM
Essential					
Arginine	2.06 ± 0.11^{d}	1.52 ± 0.03^{e}	4.32 ± 0.04^{b}	4.50 ± 0.04^{a}	3.59 ± 0.12^{c}
Threonine	0.52 ± 0.02^{d}	0.72 ± 0.02^{c}	1.48 ± 0.01^{b}	1.38 ± 0.01^{b}	1.97 ± 0.09^{a}
Valine	0.89 ± 0.01^{e}	1.04 ± 0.01^{d}	2.49 ± 0.02^{a}	2.37 ± 0.05^{b}	2.31 ± 0.02^{c}
Lysine	0.49 ± 0.03^{d}	0.64 ± 0.00^{c}	0.78 ± 0.00^{b}	0.84 ± 0.02^{b}	2.83 ± 0.06^{a}
Isoleusine	0.63 ± 0.04^{d}	0.78 ± 0.01^{c}	1.67 ± 0.01^{b}	1.71 ± 0.04^{b}	2.31 ± 0.03^{a}
Leusine	1.07 ± 0.03^{d}	1.23 ± 0.01^{c}	2.96 ± 0.02^{b}	2.91 ± 0.08^{b}	3.66 ± 0.03^{a}
Phenylalanine	0.68 ± 0.02^{d}	0.79 ± 0.01^{c}	2.00 ± 0.02^{b}	2.02 ± 0.06^{b}	2.50 ± 0.08^{a}
Tryptophan	0.11 ± 0.00^{d}	0.13 ± 0.01^{c}	0.24 ± 0.00^{b}	0.01 ± 0.00^{e}	0.52 ± 0.01^{a}
Histidine	0.27 ± 0.00^{d}	0.34 ± 0.01^{c}	0.68 ± 0.00^{b}	0.33 ± 0.01^{c}	1.26 ± 0.03^{a}
Methionine	0.26 ± 0.02^{c}	0.22 ± 0.01^{d}	0.73 ± 0.00^{b}	0.89 ± 0.01^{a}	0.74 ± 0.01^{b}
Total EAA (%)	6.98 ± 0.24^{c}	7.42 ± 0.09^{c}	17.34 ± 0.03^{b}	16.90 ± 0.22^{b}	21.49 ± 0.21^{a}
Non-essential					
Aspartic acid	1.38 ± 0.05^{d}	1.62 ± 0.02^{c}	3.38 ± 0.01^{b}	3.52 ± 0.06^{b}	4.84 ± 0.12^{a}
Serine	0.71 ± 0.00^{d}	0.78 ± 0.02^{d}	2.00 ± 0.02^{b}	1.81 ± 0.06^{c}	2.45 ± 0.05^{a}
Glutamic acid	3.44 ± 0.05^{c}	2.78 ± 0.04^{d}	8.24 ± 0.06^{b}	8.74 ± 0.10^{a}	8.60 ± 0.23^{a}
Glycine	0.74 ± 0.01^{d}	0.95 ± 0.02^{c}	1.98 ± 0.03^{b}	2.02 ± 0.07^{b}	2.14 ± 0.02^{a}
Alanine	0.59 ± 0.20^{b}	0.82 ± 0.00^{b}	1.68 ± 0.02^{a}	1.92 ± 0.11^{a}	1.80 ± 0.05^{a}
Proline	0.53 ± 0.01^{d}	0.64 ± 0.01^{c}	1.70 ± 0.02^{b}	1.66 ± 0.04^{b}	2.38 ± 0.04^{a}
Tyrosine	0.29 ± 0.03^{e}	0.46 ± 0.01^{d}	1.05 ± 0.04^{b}	0.65 ± 0.01^{c}	1.65 ± 0.06^{a}
Cystine	0.20 ± 0.01^{d}	0.19 ± 0.01^{d}	0.23 ± 0.00^{c}	0.35 ± 0.00^{b}	0.71 ± 0.00^{a}
Total NAA (%)	7.87 ± 0.31^{c}	8.25 ± 0.12^{c}	20.25 ± 0.06^{b}	20.50 ± 0.13^{b}	24.45 ± 0.61^{a}
Total Amino Acid (%)	14.85 ± 40^{c}	15.67 ± 0.21^{c}	37.60 ± 0.03^{b}	37.39 ± 0.10^{b}	45.94 ± 0.82^{a}
Total AA as % of CP	84.9	63.4	88.7	81.4	92.2

Data are presented in the mean values ± sd (n = 3). Values with different letters within same row are significantly different (p <0.05). PKC = palm kernel cake; AA = amino acid; fPKC = fermented palm kernel cake; EAA = essential amino acid; pPKC (f) = polypeptide PKC (feed grade); NAA = non-essential amino acid; pPKC = polypeptide PKC (food grade); SBM = soy bean meal; CP = crude protein

analyses of fPKC reported by Iluyemi *et al.* [5] might be due to different strain of *A. niger* and fermentation conditions. The total amino acid and true protein content are more reliable values than the crude protein.

Most of the amino acid contents increased in the fPKC compared to the values in PKC, except for arginine, methionine, glutamic acid and cystine. Normally sulfur containing amino acids (cystine and methionine) were lower since it is known that these amino acids are easily degraded by heat [22]. PKC protein also has a poor amino acid balance, with lysine being a major limiting amino acid [23].

The nutritional value of protein is considered high if the composition of its essential amino acid content is close to the essential amino acid profile required in the diet of most animals. Modification and improvement in fermentation and drying process of the fPKC should be applied to reduce the non-protein nitrogen content, such as nucleic acid, which have no known nutritional value [15]. Hydrolyzed protein shows better availability since low molecular weight peptides and amino acids are released. Therefore, higher in vivo protein digestibility values should be expected in fPKC, since there is a marked increase in free amino acids after fermentation. The increase in hydrophobic amino acids such as isoleucine, leucine and lysine was also important, due to the effects that these have on the physical and functional properties of food proteins [24, 25].

In comparison with pPKC, pPKC(f) and SBM,

protein value of fPKC was significantly lower than these three materials (Table 4). The increase in the total EAA in feed grade of polypeptide PKC (17.3 %) compared to the level in pPKC for human consumption (17.0 %) was not statistically significant. Results also showed that the total amino acid in pPKC (f) accounted for 88.7 % of crude protein compared to the level in pPKC for human consumption, which accounted for only 81.4 % of crude protein. It means that the total of non-protein nitrogen in pPKC is higher compared to the level in pPKC (f). The results showed that pPKC(f) could be used as an alternative protein source to substitute SBM in animal feed. However, it depends on the price of the material because the total amino acid in SBM was significantly higher compared to the level in pPKC(f). While for the fPKC, it was proven that the material can substitute 30 % of corn in poultry feed [7].

CONCLUSION

Results from the study showed that SSF technique could be used to increase nutritional composition of PKC. Both fPKC and pPKC (f) could be considered as potential alternative energy and protein sources for monogastric animals. Further research involving animal trials and toxicology tests will be carried out to study animal growth performance, protein quality (PER) and to determine the side effects of secondary metabolites especially in the fermented PKC, which could cause acute or chronic toxicity to the tested animal.

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Dy.Ybi.AVROS variety, which possesses many of the desirable ideotype traits, has been developed (Tables 2,3; Figs. 1,3) This variety could be planted at a higher density and consequently higher yields can be expected because of the better HI.

Future directions in oil palm genetic improvement

Breeding Objectives. Palm oil will remain as a commodity crop being essentially a food crop although its non-food uses (ca. 20%) e.g. biofuel, oleochemicals, are assuming increasing importance. Yield thus still needs to improve further. Likewise, we still need to further facilitate harvesting because of increasing labour cost. Compact palms with high HI [10], easily recognizable ripe (virescens) non-shedding low lipase fruits (inhibits free fatty acid formation and maintains oil quality) and can be planted at high density for mechanized harvesting are sought [11]. Basal stem rot disease caused by *Ganoderma boninense*, which was a problem in oil palms replanted from palms in coastal areas, is becoming a serious problem inland. Chemical and

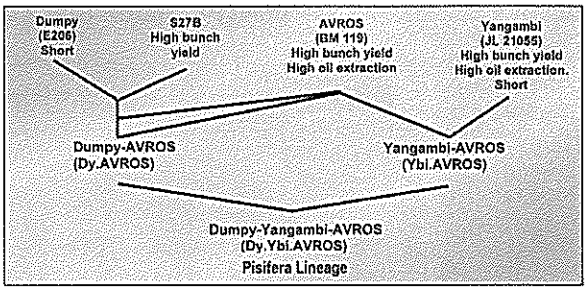

Figure 1. Breeding Dy.AVROS and Dy.Ybi.AVROS semi-dwarf varieties.

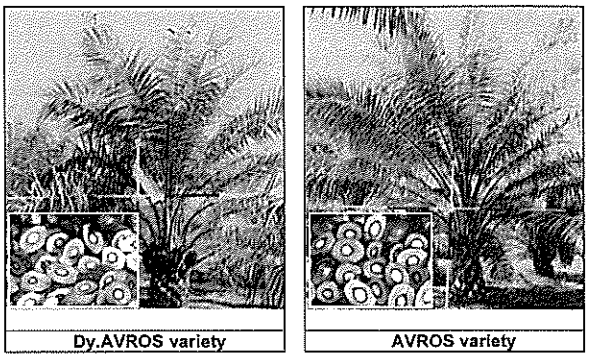

Figure 2. Semi-dwarf high yielding Dy.AVROS vs tall AVROS varieties (1 mark on pole = 30cm).

Table 2. Combined analysis of BT6B and BT6C trial results.

Pisifera Parental Lineage	No. Plots	Mean									
		Yield Component					Vegetative Component				
		FFB	BNo	BW	OB	OY	HT	LAI	FL	FW	FA
Dy. AVR (sibbed)											
OBS20/30	29	17.7	15.3	8.3	26.7	4.74	45.9	6.4	425	2.4	11.1
053/20	16	18.4	12.7	10.7	24.6	4.52	49.4	6.8	449	2.4	11.7
053/27	33	18.8	15.1	9.1	24.6	4.70	45.3	6.8	455	2.5	11.9
053/33	8	17.6	16.1	8.1	27.1	4.76	45.1	6.4	425	2.3	11.1
	Mean	18.1	14.8	9.1	25.8	4.68	46.4	6.6	439	2.4	11.5
Dy. Ybi. AVR											
0138/1	10	17.9	13.5	9.8	27.7	4.98	43.2	6.8	452	2.1	11.0
0138/4	2	20.3	15.5	9.4	26.2	5.36	49.4	6.6	465	2.6	11.8
	Mean	19.1	14.5	9.6	27.0	5.17	46.3	6.7	459	2.4	11.4
0158/1	6	21.2	17.1	8.8	25.7	5.53	57.9	7.0	448	2.3	11.9
0158/15	5	20.8	20.8	6.8	25.4	5.34	53.9	6.0	428	2.2	10.7
0158/16	12	17.3	15.6	8.0	27.7	4.88	46.3	6.6	443	2.5	10.8
	Mean	19.8	17.8	7.9	26.3	5.25	52.7	6.5	440	2.3	11.1
Control											
AA DxP	6	18.1	15.5	8.3	26.8	4.88	51.3	6.5	441	2.3	10.8
GH DxP	6	19.1	17.1	8.2	27.1	5.25	61.7	6.8	453	2.2	11.2
MSE (s^2)		7.2	5.2	1.4	1.7	.52	87.4	0.55	489	.05	1.5

Yield Component: 37–60 months after field planting (MAP), FFB – fresh fruit bunch yield (t/ha/yr), BNo – average bunch number (no/p/yr), BW – average bunch weight (kg), OB – oil to bunch (%) OY – oil yield = FFB x OB (t/ha/yr)

Vegetative Component: BT6B (59 MAP other than Ht: 60 MAP), BT6C1(54 MAP), BT6C2(57 MAP other than Ht: 49 MAP)

HT – palm height (cm), LAI – leaf area index, FL – frond length (cm), FW – frond weight (kg), FA = frond area (m2).

Table 3. Combined analysis of BT10A and BT10B trial results.

Pisifera Parental Lineage	No. plots	Mean											
		Yield Component					Vegetative Component						
		FFB	BNo	BW	OB	OY	HT	LAI	FL	FW	FA	FP	GIR
Dy AVR (sibbed)													
OBS20/26	16	13.0	13.0	7.2	26.9	3.5	37.6	5.0	395	2.1	9.4	21.9	264
OBS20/30	4	10.7	10.6	7.0	30.1	3.5	38.9	4.9	392	2.4	9.3	21.3	276
053/9	19	12.3	11.4	7.7	29.2	3.6	37.1	5.1	397	2.1	9.4	22.5	264
053/27	13	12.3	11.1	8.1	27.4	3.4	36.5	5.5	410	2.2	9.8	21.7	276
	Mean	12.1	11.5	7.5	28.4	3.5	37.5	5.1	399	2.2	9.5	21.9	270
Dy Ybi. AVR													
0138/1	15	12.6	12.5	7.2	28.6	3.7	38.1	4.8	381	2.0	9.4	22.7	257
0138/21	3	12.9	12.3	7.5	31.1	4.0	43.9	5.3	394	2.1	9.7	22.6	266
	Mean	12.8	12.4	7.4	29.9	3.9	41.0	5.1	388	2.1	9.6	22.7	262
0158/1	8	15.0	15.5	7.0	31.5	4.8	42.9	5.5	382	2.0	9.9	24.5	270
0158/9	22	14.1	16.9	6.0	30.2	4.3	45.7	5.1	377	2.0	9.1	23.9	264
0158/20	10	12.2	12.3	7.1	28.1	3.4	45.1	5.2	384	2.0	9.2	24.3	272
	Mean	13.8	14.9	6.7	29.9	4.2	44.6	5.3	381	2.0	9.4	24.2	269
GH DxP Control	8	14.7	14.8	7.2	28.3	4.2	51.2	5.6	406	2.1	9.8	24.6	266
MSE (s^2)		9.0	6.8	0.7	3.9	.67	39.2	.3	252	.03	0.6	1.2	76

Yield component: 37-54 MAP (months after planting), FFB – fresh fruit bunch yield (t/ha/yr), BNo – average bunch number (no/p/yr) BW – average bunch weight (kg), OB – oil to bunch (%), OY – oil yield = FFB x OB (t/ha/yr)

Vegetative component: 54 MAP, FP (43 – 55 MAP)

HT – palm height (cm), LAI – leaf area index, FL – frond length (cm), FW – frond weight (kg), FA = frond area (m2), GIR = girth (cm).

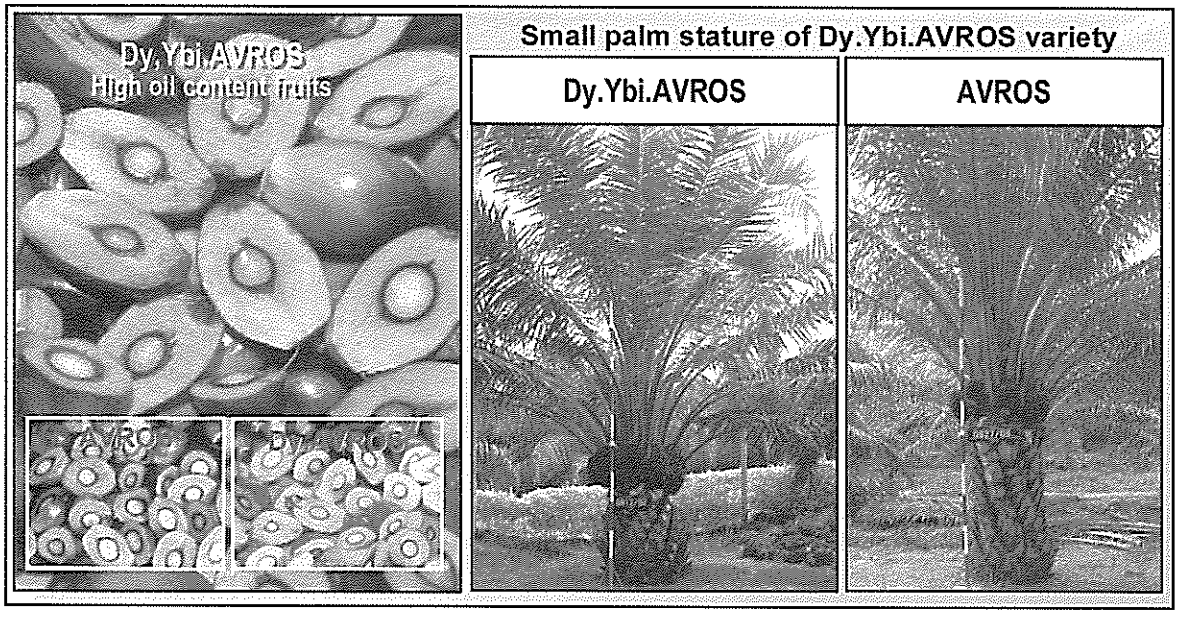

Figure 3. Breeding semi-dwarf Dy.AVROS and Dy.Ybi.AVROS high harvest index varieties. Note the progressive reduction in trunk height (1 mark= 30 cm) and canopy size and increase in bunch yield and fruit mesocarp thickness from the tall AVROS, to the semi-dwarf Dy.AVROS and the high harvest index Dy.Ybi. AVROS varieties.

cultural control methods are ineffective. Resistant varieties offer the best solution and resistance sources are available [12-14]. All these objectives are geared towards high yield and lower production cost.

Palm oil has many uses [15]. Its biosynthetic pathway can be genetically manipulated to produce a host of oils with different fatty acid compositions e.g. high oleic acid, high stearic acid, high ricinoleic, high lycopene, high palmitoleic and high polyhydroxy butyrate (PHB) [16]. These are the value added traits targeted for genetic improvement in MPOB (Malaysian Palm Oil Board). High oleic acid makes palm oil unsaturated thus hypocholestrolemic, lowering cardiovascular heart disease risk when consumed [17], making it more liquid for the salad and cooking oil market in temperate countries and also as a good feedstock for oleochemical industries. Stearic acid is used as cocoa butter substitute and also in personal healthcare products e.g. lotions, creams. Lycopene is a useful nutraceutical and palmitoleic acid is used in pharmaceutical applications for its anti-thrombotic attribute. PHB can be made into biodegradable high tensile bioplastics. Ricinoleic acid has use in high grade lubricants besides cosmetics and pharmaceuticals. Carotenes and tocopherols/tocotrienols with antioxidant properties found in palm oil can be used to make valuable vitamins A and E health supplements.

Breeding Progress. Dwarf palm breeding began in the late 1950s, but the Dy.AVROS semi-dwarf variety was released in the early 1980s and the Dy.Ybi.AVROS variety has become available only recently. Breeding is a slow process with a perennial tree crop such as the oil palm which needs about eight years to turn over a breeding generation. Many of the extreme traits, both agronomic and value-added, are also likely to be found in semi-wild or alien species making the breeding process to incorporate these traits into modern high yielding cultivars even more protracted. Since the advent of modern breeding, breeders have always been seeking means to expediting this. Biotechnology offers this great opportunity.

Biotechnology. Biotechnological tools can assist greatly in all the three distinct stages in the breeding process: creation of genetic variation; selection of the desirable genetic variants; stabilization and propagation of the desirable variant genotype as the commercial variety. Transgenics or GM can widen genetic variability by the incorporation of desirable genes from distant or alien species including bacteria and animals. Marker assisted selection (MAS) enables early and more efficient selection. Tissue culture enables clonal propagation of elite hybrid genotypes as commercial varieties, or of the parents of elite hybrids for clonal hybrid production. Dihaploid (fully homozygous) parents, equivalent to fully inbred parents, of elite hybrids can also be developed via tissue culture.

Tissue culture clonal propagation in oil palm is perhaps the most developed technology-wise (Figs. 4,5). The impetus for this development is the existence of significant genetic variability within the commercial variety comprising mixed hybrids from non-inbred parents [18]. Thus individual palms or genotypes can yield considerably more than the mean of the hybrids. Cloning can propagate the superior genotypes much faster than by hybrid breeding which would take 20-30 years. Tissue culture is the only means to clone the oil palm

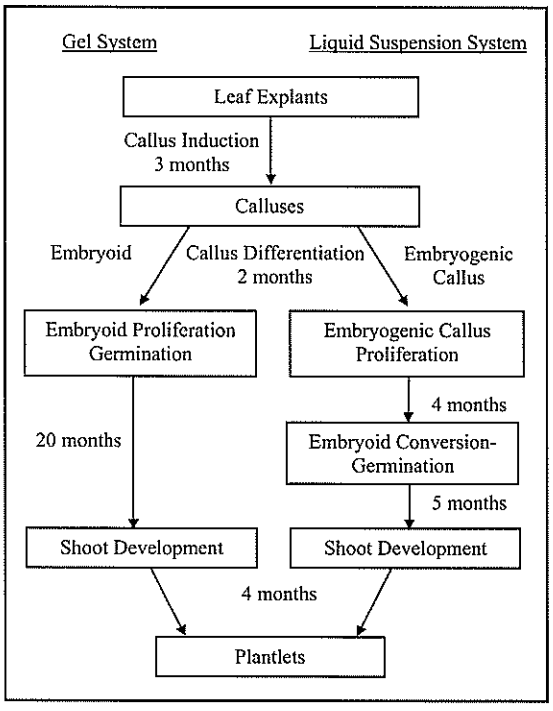

Figure 4. Oil palm tissue culture stages and duration in gel and liquid suspension systems