

CLONAL MICROPROPAGATION OF OIL PALM (*ELAEIS GUINEENSIS* JACQ.)

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ABSTRACT

Dami Oil Palm Research Station is home to the only commercial Tissue Culture laboratory in Papua New Guinea. The laboratory affords New Britain Palm Oil the option of cloning elite seed palms in order to maximise production and to provide germplasm security. The laboratory was set up in December 1996 and has gone on to produce 1300 palms representing 37 clones. In April 2004 the laboratory and the procedures used at Dami were accredited with the International Standards Organisation Environmental Management standards (ISO 14001). This paper details the Chemical and Biological aspects of these procedures and also describes the physical requirements necessary to operate the Dami Tissue Culture Laboratory.

INTRODUCTION

Tissue culture is often referred to as the art and science of growing plant parts into whole plants. This is achieved under aseptic conditions *in vitro* or 'in glass'. (George 1993). Examples of the different plant parts (explants) used in tissue culture are:

1. Plant culture – the culture of seedlings or larger plants.
2. Embryo culture – the culture of isolated mature or immature embryos.
3. Callus culture – the culture of tissue arising from explants of plant organs.
4. Suspension or cell culture – the culture of isolated cells or aggregates in a liquid medium.
5. Protoplast culture – the culture cells devoid of their cell wall

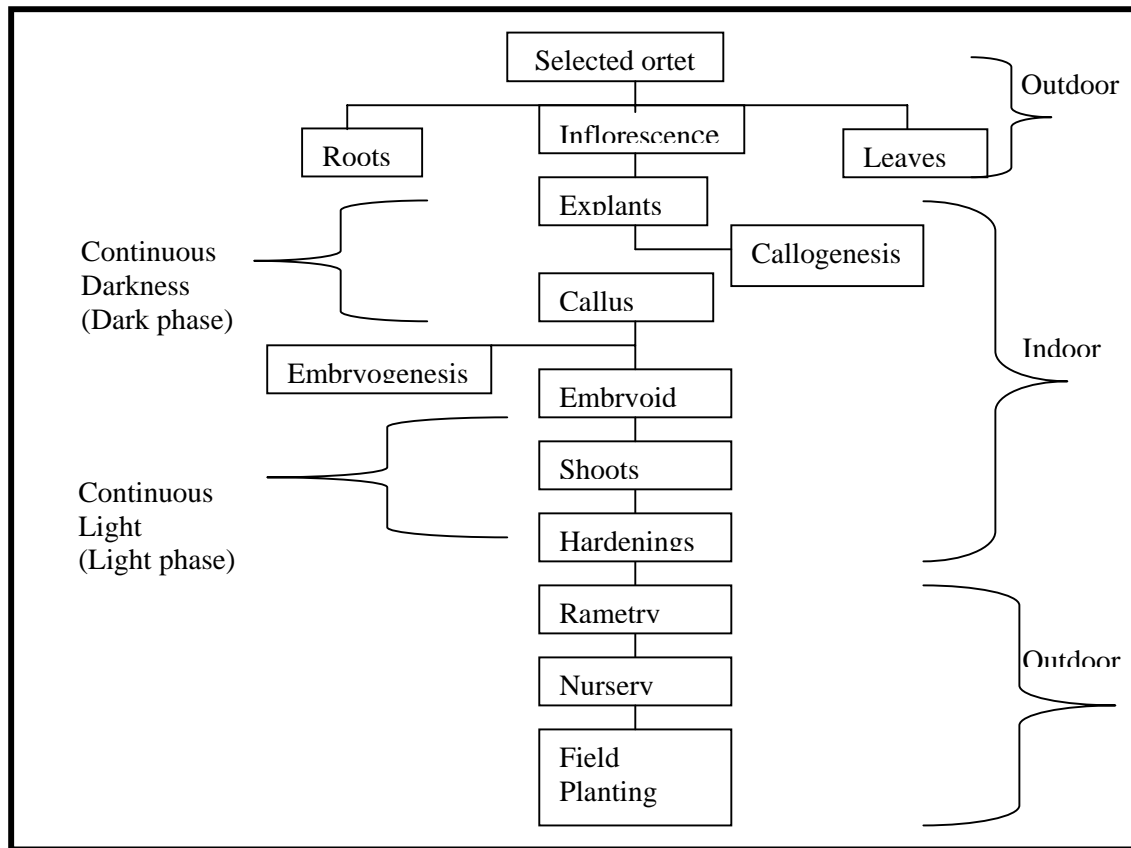
For oil palm there are no *in vivo* (in life) methods available for vegetative propagation. Oil palm does not produce offshoots, as all the auxiliary buds form inflorescences and there are no existing methods for the establishment of cuttings.

Tissue culture has made it possible to clonally propagate palms and so mass produce uniform planting material. The tissue culture technique for oil palm was developed in the 1970's, a decade after it was well established for other crops. The oil palm industry has been quick to capitalise and commercialise this new technology.

Prior to the establishment of a dedicated tissue culture laboratory at Dami, all material was sent to Unifield in England for propagation. When the palms were sufficiently advanced they were sent back to PNG for field planting and evaluation. This arrangement proved to be unnecessarily complex and the practice ceased by the late 1990's when New Britain Palm Oil Limited established the Dami tissue culture laboratory.

This paper will provide some insight into the complex world of tissue culture. It will address the biological, chemical and physical aspects of oil palm tissue culture at the Dami Oil Palm Research Station.

Figure 1: Stages in oil palm tissue culture



BIOLOGICAL PROCESS OF TISSUE CULTURE

As can be seen in Figure 1 the actual process of oil palm micro-propagation has relatively few steps but the process is by no means simple.

Approach to Sampling

The most responsive parts of the oil palm in regard to tissue culture are those areas which are meristematic (rapidly dividing) and young. Such areas are found in roots, flowers and in the crown itself. These areas provide the best starting material (explants). Ideally the explant should provide large numbers of starting tissues and have little or no contamination. While it is possible to propagate oil palm from young roots, leaves and crown tissue, Dami uses immature inflorescences. Inflorescence sampling eliminates the problems associated with disease and palm damage common to the other explant tissue types, provides a reasonable number of explants (although less than leaf sampling) but more importantly the sampling procedure causes minimal damage to the breeding palms.

Donor palm (ortet) selection

The biological process of tissue culture starts with the selection of the donor palm. At Dami, propagation of oil palm is primarily a tool for the plant breeder. Palm selection is based on a range of characteristics such as yield, extraction rates, height and disease or drought tolerance. These palms are

graded and a list submitted to the tissue culture laboratory for propagation (clonal propagation). Plant tissue aims to clone a minimum of 50 grade 1 palms, 40 grade 2 and 30 grade 3 suitable for planting in the field.

Details of the selected ortet are added to a computer control program. This entry is the start of the management program for recoding ortet and clone performance.

Palm Sampling and explant processing

The inflorescence is protected by an inner and outer spathe, which helps in minimising potential contamination. The optimum inflorescence tissue is obtained from leaf axils (frond) 8, 9 or 10. It is important that the correct frond number be identified prior to removal of the inflorescence. The inflorescence is carefully removed with the aid of a car jack and a specially designed, curved knife. A procedure originating in Dami that ensures that the donor palm and the inflorescence spathes are not damaged in any way during the extraction process. As soon as the inflorescence is removed from the palm it is sprayed with 80% ethanol, wrapped in tissue paper and placed inside a sampling bag for protection. The inflorescence is then taken immediately to the laboratory for processing.

The spathes are removed from the inflorescence in the dissecting or plant preparation room and the inflorescence soaked in a 0.35% active ingredient NaOCl (household bleach) solution for 5 minutes. The inflorescence is then transferred to 80% ethanol for 5 minutes. The inflorescence is cut into quarters and each quarter placed in a jar of culture medium designed to test for contamination. If the sections are not contaminated they are then cut into smaller pieces (2 to 4mm) and placed on callus induction media containing auxin (plant growth substance) to initiate callus (unorganized mass of cells) development. In oil palm, most callus is initiated from the explants by 12 weeks, however this can take up to 24 weeks.

The number of explants that can be obtained from an inflorescence varies, inflorescences from frond 8 are approximately 116mm long and provide 178 explants, frond 9 is 160mm and 210 explants, while frond 10 is 177mm and can supply up to 250 explants. The percentage contamination during the dissecting process for this laboratory is less than 10%. The explants are now ready for regeneration culture in the dark room at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Explants

The explants are initially placed on a screening media for one week to ensure that they are free from contamination. Then the explant tissue is transferred to explant media and transferred to fresh media every 12 weeks for a total of 36 weeks. The culture medium supplies all the micro and macronutrients required to maintain explant survival alive and continue development. Auxin in the form of NAA is added to the media to stimulate callus initiation. The media recipe is confidential and no details of this or any of the other medias used will be given

The average callus production from explants is approximately 5%. The explants are kept in continuous darkness as this minimises phenolic production that would inhibit further development.

Once the explants have been transferred to the explant media the transaction is recorded on the data input sheet and the information in the computer updated. All subsequent transaction for these pieces of tissue can now be tracked. For each stage in the culture process strict standards for biological material are maintained.

Callogenesis

Callus is induced in the explants and these cells can eventually divide and differentiate into specific tissue to produce shoots and roots. Callus is undifferentiated tissue which has the ability to give rise to unspecialised cells that are meristematic. Addition of 2,4-D to the media then triggers these cells to produce embryoids (Plate 1).

Callus is normally treated to two cycles on induction culture media (16 weeks) before being transferred to two cycles of proliferation media for another sixteen weeks (Table 1). Callus will follow this cycle until embryoids are produced. The embryoids are then transferred to the light culture room for further development. Callus cultures are kept in continuous darkness for up to two years before being discarded. The average percentage of embryoid production from callus is 3% and these are generally produced after six months at the callus stage.

Plate 1 Embryoid initiated from callus.



Embryogenesis

Embryogenesis can take two forms; embryos can be produced directly or indirectly where the explant goes through callus stage. Indirect embryogenesis is more common (George 1993). While direct embryogenesis is more desirable, it is more difficult to achieve as the conditions necessary are more critical than those required to produce embryogenic callus. One problem associated with indirect embryogenesis is the potential for genetic variation, especially where callus has been subjected to prolonged periods in culture. Oil palm uses the indirect embryogenesis method.

When embryoids are produced they are transferred to embryoid media and are transferred from continuous dark culture to continuous light culture. Again all transactions are recorded on the one input sheet and the computer database updated.

The embryoid stage marks the beginning of the light phase of the oil palm tissue culture process (Figure 1). Embryoids are multiplied until the total number reaches the required 350. Of these 350 embryoids only 250 will produce shoots (shooting percentage of 75%).

Table: 1 Tissue types and residency periods of oil palm tissue cultures at Dami

Type of Culture	Stage of Development	Transfer Interval	Period in each stage
Explant	Callus initiation	12 wks	36 wks
Callus	Multiplication	8 wks	104 wks
Embryoid	Multiplication and Shoot product	8 wks	48 wks
Shoots	Root formation on shoots	8 wks	16 wks
Hardenings	Development of a hardy plant	8 wks	32 wks

Shoot and root production

The introduction of cytokinin (plant growth regulator) into the culture medium is now required for the production of shoots. When the shoots are over 4cm in height they are transferred to a culture medium which encourages root development and growth. In both instances the developing plant is kept on the culture media for a period of 16 weeks and transferred to fresh media at eight weeks intervals. Plantlets that have no evidence of a terminal inflorescence, have roots 5mm long and are over 6cm in height are transferred to a hardening media for the acclimatization phase (Plate 2).

Plate 2 Shoots (A) after 8 weeks on rooting media and plantlet (B) after 16 weeks on hardening media



A



B

Hardening

Hardening is the last stage before the plantlets are transferred to the natural environment (Figure 1). Hardening is kept for thirty two weeks in the light culture room and are transferred to fresh media which is free of hormones and has reduced carbohydrate levels every eight weeks (Table 1). Only those plantlets which are sturdy, have shoot development over 11 cm height, have with no evidence of a terminal inflorescence and good root development are transferred to the pre-nursery stage.

Pre-nursery (Rametry) and Nursery

This is the stage where plants from tissue culture are gradually exposed to outside environment. The young palms are planted into poly bags and placed inside a protected environment.

A high humidity level is maintained during this phase while the young palms plants develop leaf epicuticular wax which does not develop under the conditions provided in the tissue culture process. The epicuticular wax is necessary to ensure the plants do not lose excess water when exposed to the outside environment. Another reason for increased protection is that plants in tissue culture have been provided with all their carbohydrate requirements and have not had to depend on photosynthesis for the supply of energy. The young palms are kept in the rametry for fifteen weeks and then for another fifty two weeks in an outside nursery before they are planted in the field.

Flower abnormality

One of the setbacks for oil palm clonal propagation has been the development of abnormal flowering after planting in the field. Out of the 12 clones produced and are in production so far, 5 have shown floral abnormalities. Abnormal flowering is somaclonal variation with genetic, epigenetic and chemical (possibly 2, 4-D) causes. It has been suggested that the longer explant tissues are kept in culture, especially at callus stage, the more likely it is that somaclonal variation will occur.

CHEMICAL PROCESS OF TISSUE CULTURE

Plant nutritional requirements vary between explant types and between plant species. As plants *in vivo* require soil for support and nutrients from the soil for growth and other metabolic activities, tissues *in vitro* are supplied with nutrients from artificial medium for growth and development. Medium used for oil palm tissue culture is no exception and must contain all the macro and micro elements, organic supplements such as vitamins and amino acids necessary for plant growth. Each developmental stage has its own specific media requirements which encourage growth and also initiate the next stage of development e.g. explants to callus stage (Table 1).

Macronutrients

Macronutrients are elements required in larger amounts and they include: nitrogen, phosphorus, magnesium, potassium, calcium, and sulphur (William 2004).

Nitrogen is supplied as nitrate (NO_3^-) or ammonium ions (NH_4^+). These ions can be supplied from a variety of sources such as potassium nitrate (KNO_3), ammonium di-hydrogen orthophosphate ($(\text{NH}_4)_2\text{H}_2\text{PO}_4$), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) and ammonium nitrate ($(\text{NH}_4)\text{NO}_3$). Nitrogen is an important element for the growth and development of plant cells or tissues *in vitro* (George 1993).

Phosphorus is supplied as phosphate ion PO_4^{3-} and is involved in transfer of energy within the cell and regulates the activities of many enzymes. It is also a component of biological molecules such as DNA and RNA. Phosphorus is supplied as ammonium, sodium or potassium di-hydrogen orthophosphate salts ($\text{NH}_4\text{H}_2\text{PO}_4$, $\text{Na}_2\text{H}_2\text{PO}_4$ or KH_2PO_4 respectively).

Potassium is supplied as chloride, nitrate or orthophosphate salt and is important in cellular homeostasis like pH and osmotic regulation. Phloem transport and stomatal guard cell turgor rely on potassium for its ability to be transported across plant cell membranes (Rayns and Fowler 1993).

Magnesium is supplied as magnesium sulphate (MgSO_4) and is an important enzyme co-factor. It can also fulfill some of the pH and osmotic regulatory functions of potassium.

Calcium is supplied as calcium chloride (CaCl_2). It is an important enzyme co-factor and enzyme regulator. It is also involved in establishing the structure and properties of both cell membranes and cell walls (Bhojwani and Razdan 1996).

Sulphur is supplied as magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and is an important part some amino acids. It is also part of some enzyme co-factors.

Sucrose provides the main source of carbohydrates used for oil palm tissue culture in this laboratory.

Micronutrients

Micronutrients are elements required only in small quantities and include: iron (Fe), manganese (Mn), copper (Cu), cobalt (Co), boron (B), molybdenum (Mo), zinc (Zn), and iodine (Rayns and Fowler 1993).

Iron is the most important micronutrient and is required for the formation of several chlorophyll precursors. It is also needed as part of protein development to undertake oxidation and reduction reactions.

Manganese is required in lesser amounts than iron as cultures in vitro are not autotrophic. The role of manganese is mainly to maintain chloroplast ultra structure and for photosynthesis (Rayns and Fowler 1993).

Copper is essential for chlorophyll synthesis and photosynthetic electron transport. It involves in enzymatic activity.

Cobalt is essential for coenzyme assimilation and boron also plays an important role in the development and growth of new cells.

Molybdenum is an essential component of an enzyme involved in catalysing the conversion of NO_3 to NO_2 .

Zinc is essential in the synthesis of auxin and in enzyme systems for plant growth.

Iodine is included in culture media because of its role as a reducing agent. It is suggested in George 1993 that it improves root growth and contribute to survival and growth of cultures of some species.

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Gelling agents

Soils provide support for plants and likewise agar provides that function for tissue culture plants. Agar is extracted from seaweed and is the most commonly used gelling agent for plant tissue culture. It is insoluble in cold water and produces gels that melt at temperature around 100⁰C and solidify at temperature around 45⁰C. The hardness of the medium depends on the concentration of agar and pH of the medium used. Generally concentrations of between 0.6 to 0.8% (weight/volume) are used in most crops. In oil palm the concentration varies with the different stages of culture and the range of concentrations goes from 0.8 to 0.35% as a result. For example the explants require a firm media (0.8%) whilst the rooting stage and onwards grow better in more liquid gels (0.35%).

Two main gelling agents used in oil palm tissue culture are agar (a natural sea weed derivative) and phytigel (a synthetic matrix designed especially for plant culture). Phytigel is used only for the explant media stage while agar is used as the gelling agent for the other culture stages.

Plant growth regulators

Plant growth regulators (PGRs) play a major role in cellular differentiation in plants in the natural state. In plant tissue culture PGRs are required to drive the tissues from one stage to the next, an example is the role played by auxin (2,4-D) in the production of embryos from callus cultures. While known amounts are supplied, the cells or tissues also produce PGRs internally and this can complicate the issue (William 1979).

Auxins are used for their promoting effects and cell elongation. 2,4-D, and NAA are some common auxins used in oil palm tissue culture.

Cytokinins are also used for promoting growth and the ones common to oil palm tissue culture are zeatin, adenine and 6-benzillaminopurine (BAP).

Activated charcoal is used in the media for its metabolite absorbing abilities. It is used for removing toxic metabolites from plant cultures and can enhance rooting by reducing illumination of the submerged parts of the culture (Rayns and Fowler 1993). In oil palm tissue culture, it is used in the callus stage media.

PHYSICAL PROCESS OF TISSUE CULTURE

Irrespective of the laboratory size, the following features are basic necessities and considered essential for any successful tissue culture

- 1 Media preparation room
- 2 Transfer room
- 3 Culture rooms

Additional rooms such as explant preparation, storage and generator set housing are also necessary for operating in the PNG environment and to ensure the successful transfer of oil palm plantlets from the tissue culture laboratory to the nursery, an additional requirement is the construction of a rametry (or pre-nursery) shade house. These 7 element form the basis of oil tissue culture at Dami OPRS (Appendix 1). The laboratory also contains an office, a conference room, and toilet and shower

facilities. The latter two add to the comfort of the work force but also limit the frequent need to exit the building and provide hygiene areas where cleanliness can be enforced. Both contribute to the overall cleanliness of the facility

Laboratory requirements

A good design should have an internal layout governed by workflow patterns, it should maximise clean work practices and promote minimal back tracking of successes and errors (Lord and Eeuwens 1995).

Many tissue culture laboratories are constructed within existing buildings, however if resources and finances allow, a freestanding structure is a better option. It is easier to design out problems than manage existing ones. It has been the experience in many other laboratories that within a well designed laboratory it is easier to take corrective measures when problems arise. An example of this can be seen in maintaining cultures free of contamination. It is much easier to minimise human traffic within a separate building than it is to control movement when a laboratory is contained within a building. It also makes the planning of distinct, unclean areas (contaminated field material) from clean areas (free of contamination) easier.

The Dami tissue culture facility is a freestanding structure and has all the features required by a well design laboratory. It was purposely built and designed to contain many feature to limit microbial contamination and reduce cost.

Reducing the microbial challenge

Overall contamination levels have been minimal, less then 5% since the establishment of the laboratory. The internal layout is such that, the cleaner areas such as transfer and culture rooms are located away from the main entrance and areas of unclean activities (plant preparation and washing up areas).

Another important feature of the Dami laboratory design is the insulation of growth rooms to ensure energy conservation and reducing overall running expenses. The culture rooms themselves are constructed from solid blocks of polystyrene sandwiched between steel plates and covered in food grade plastic laminate.

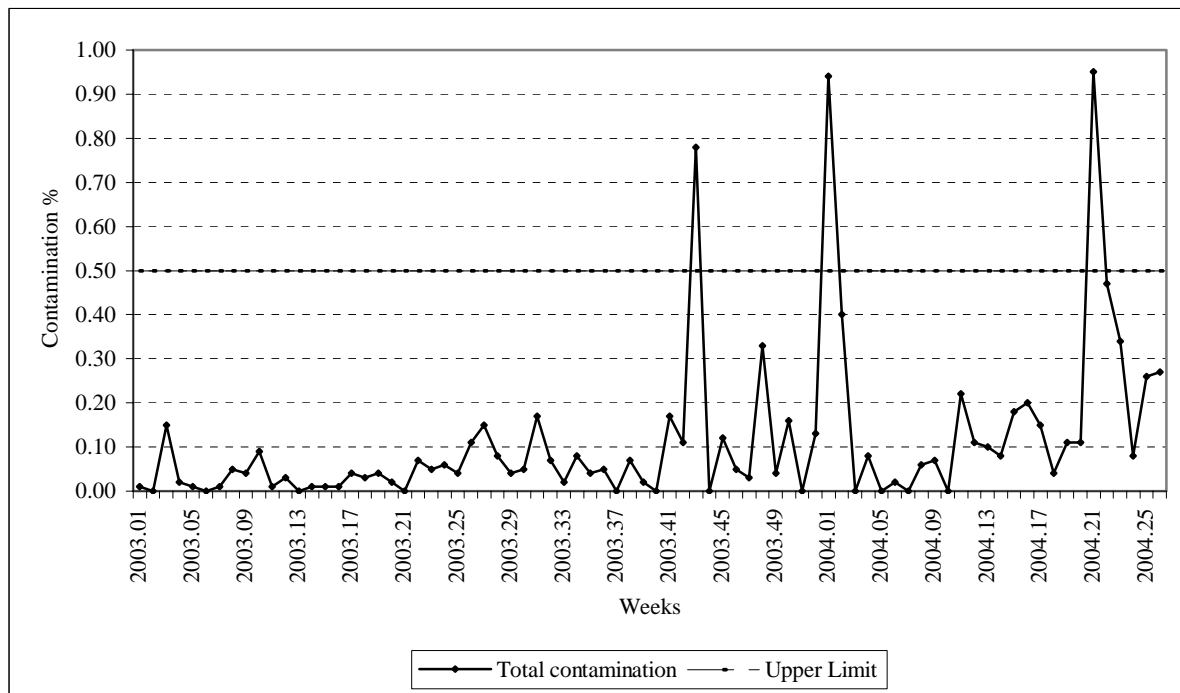
This gives excellent insulation and an easily cleaned surface capable of withstanding harsh cleansing agents.

The laboratory is fitted with air locks throughout to assist in minimising the entry of potentially contaminating organism. The laboratory has also been designed so that the internal temperatures are lower then ambient external temperature and this creates a positive air pressure such that there is a net flow of air out of the building helping to minimise the entry of contaminants (Figure 2).

Further to this sticky mats are placed at critical points to minimise entry of potential contaminants via footwear. These measures with clean standard work practice help minimise the total contamination level to less then 5 percent for most of 2003 and 2004 (Figure 2).

Laboratory waste from the tissue culture facility is segregated, with organic materials composted and hard waste being buried in landfills. In addition mercury based sterilants are banned from use within the laboratory and no prophylactic insecticide treatments are added to the media. .

Figure 2 Contamination level for 2003 – 2004



Explant Preparation Room

This room is sometimes referred to as the dirty room where plant samples are prepared for initiation into culture. Essential equipment for the explant preparation room is a dissecting bench, a laminar flow cabinet, and a washing sink. The plant material is brought into this room and then decontaminated prior to entering the rest of the building. Contamination levels are likely to be high and as a result additional filtration is provided in the air conditioning. Material is dissected and placed in the first stage media which act as a quality check on sterility.

Media preparation room

The activities associated with media preparation are undertaken in this room. Activities involve weighing and mixing of culture medium components, pH determination and media sterilisation.

It is important for the design of this room to be governed by workflow. This is evident in the current arrangement where the preparation bench is located directly below the chemical storage area with the fridge at arms length away. The dispensing area is next to media mixer, with capping off of test tubes (last activity for media making before autoclaving) performed at the end of the bench. This arrangement is very convenient for the technicians as their movement are confined to the working bench.

Equipment found in the media preparation room includes: water distilling equipment; top pan and analytical balances; pH meters; a media dispenser; a washing machine; autoclave and refrigerators. In addition a variety of ancillary equipment such as domestic pressure cookers, kettles, hot plate stirrers and a commercial soup mixer used to heat culture media prior to dispensing. Large size sinks are situated in this room for washing up. The largest single piece of equipment is the autoclave (steriliser) and this is housed in a separate area, as the heat generated during the sterilisation procedure would overwhelm the internal air conditioning. Distilled water forms the basis of most tissue culture media

(95% by volume) and to make one litre of distilled water over 60 litres of cooling water are required. At Dami this water is recapture and recycled in an effort to make best use of resources.

Media is made in batches of 15 litres, which is sufficient to fill 1500 test tubes with 10 mls each. Each batch is uniquely identified using an alpha- numeric code, which is placed on each of the stainless steel media trays (50 test tube capacity). In fact the whole laboratory was designed around these trays such that the autoclave was custom built to hold one batch of media, the trolleys which are used to move the media around also are designed to hold 30 tray (1500 test tubes) and the culture room shelving is also configured to make maximum use of these trays.

Media storage room

Once prepared the culture media is wheeled on trolleys into the media storage room. Here it is incubated to determine if the batch has been contaminated during the preparation process. This practice ensures that subsequent cultures are free of contamination and is one of many quality checks that each batch will receive during its residency in the laboratory. Media for the different culture stages in oil palm are kept on shelving for one to three weeks before they are used. Prepared media is not kept longer than one month. All surplus media is decontaminated before disposal. The media storage room has easy access both from the media preparation room and the transfer room to minimise traffic and has specific temperature and humidity controls to prevent dehydration of the media. A white board is also provided in the media storage room to record media stock levels on weekly basis and ensure that old media is not left in the room to serve as a contamination source.

Store room

This is a room within the tissue culture laboratory dedicated to the storage of all chemicals and equipment spare parts. Most items needed for tissue culture of oil palm in Papua New Guinea must be imported from overseas, especially Australia and England and as such this room is vital for our operation. Being located within the building ensures minimal disruption, easy access and ensures cleanliness is maintained.

Detailed and advance planning is required to ensure operations flow smoothly and are not disrupted by the lack of necessary stock. In PNG the unexpected can and does happen and the chemical stock carried by the laboratory is much larger than say a Malaysian or Australian laboratory. A card stock control system is in place to ensure items are reordered when required. As items are removed the corresponding card is also removed which means stock control can be done very quickly and efficiently by counting the remaining cards.

Transfer Room

This is the 'nerve centre' of tissue culture operation where tissues from all culture stages from explants to hardenings are transferred to fresh culture media. The transfer room is located away from the unnecessary human traffic and the unclean areas of the laboratory. It is equipped with laminar flow cabinets, metal trolleys for moving cultures and observation benches. A microscope is provided to inspect cultures for mite contamination before being transferred to fresh media.

Laminar airflow cabinets maintain sterile working conditions by passing a constant stream of filtered air across their work surfaces and so creating a physical barrier to microbes. The cabinets are generally free standing but require small side trolleys and good seating. Each laminar flow cabinet is equipped with a bacti-incinerator (a small electrically powered steriliser) which when used ensure culture tools are free of contaminating organisms.

Media from the store is wheeled out as required. Each operator then begins the days shift work by 'transferring' the required tissue from old media to new media. This is done strictly to a prescribed transfer interval, which varies between tissue types. The process is controlled and monitored by a single input document that the operators fill in. This transfer sheet is checked by the supervisor after each clone has been transferred and the sheets collected and fed into the central computer which provides stock control, quality assurance and performance statistics to assist the Tissue culturist in charge to manage the process.

Culture Storage Room

The plant growth rooms provide 24 hours life support for cultures to grow and multiply. The temperature (dark culture room $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, light culture room $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$), humidity ($65\% \pm 5\%$ for both rooms) and light intensity (approximately $50\mu\text{mol m}^{-2} \text{s}^{-1}$) is maintained at a constant level.

Three banks of shelving is provided in each room with fluorescent lights provided for each shelf. Air distribution socks are provided under each shelf to evenly distribute fresh air within the growth rooms. Temperature and humidity is displayed on a control panel situated outside each of the culture rooms. Backup monitoring of the temperature and humidity levels for each room is also undertaken. Pre embryogenic cultures (explant and callus) are kept in 24 hour darkness while post embryogenic cultures (embryoids, shoots and hardenings) are kept in 24 hour light. All cultures are in 20mm test tubes and stored in 150 x 310mm trays capable of holding 50 test tubes each. Each of the rooms can hold up to 60,000 cultures at any one time, however a typical population is around 47,000 at any one time. At present the population level is less than 25,000 as new selection have yet to be processed.

CONCLUSION

Over 1000 palms from 38 clones ranging from 16 to 30 plants per clone have now been field planted. A total of 342 palms from 12 clones were planted in 2001 and are now in production while 26 clones were planted in 2003. The laboratory has begun cloning ganoderma resistant material and has a number of *tenera* clones have been selected for inclusion in the cloning program. In addition the laboratory has embarked on a program to produce double haploids and is looking at adopting suspension culture as a means to scale up the production capacity.

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Appendix 1 Tissue culture laboratory layout and production flow

