SYSTEMATIC ANALYSIS OF REPRODUCTIVE DEVELOPMENT IN NORMAL AND MANTLED OIL PALM (*ELAEIS GUINEENSIS* JACQ) FLOWERS AND FRUIT

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ABSTRACT

Oil palm (*Elaeis guineensis Jacq.*) is the most efficient oil crop in the world; it uses substantially less land and resources and produces more oil than any other oil crop. Even so, to meet the growing palm oil demands due to the increasing global population, per capita consumption rates and biofuel demands, ground-breaking strategies for agronomic and genetic improvement of the commercial planting material are necessary. Clonal propagation through tissue culture has proven to be useful in producing uniform planting materials. However, there are incidences of the deleterious floral homeotic mutant, mantled, in oil palm ramets.

In this study, standardised protocols and analytical parameters for the extraction and characterisation of oil palm inflorescences, bunches and pollen in the context of the mantled abnormality are proposed. Genotyping using twenty SSR markers showed good discriminatory powers and revealed ten 'off types'. Methylation detection at the *EgDEF1 KARMA* locus using *RsaI* showed an 18.75% error in distinguishing mantled from normal. Thus, accurate phenotyping and appraisal of mantled phenotype were achieved through visual scoring of unripe bunches. This novel phenotyping regime allowed quantification of the severity as well as variability associated with the aberrant phenotype.

For selection and extraction of comparable inflorescence samples from normal and mantled ramets, a new developmental classification was formulated, and the field sampling and histology protocols were optimised through trial. The different developmental categories were validated using ANOVA (F probability<0.001) and Fisher's protected least significant difference test. This developmental classification supplements the previous model for developmental stage prediction and enables precise field identification of key developmental events. Subsequently, a reproductive developmental series for oil palm from early inflorescence development to floral maturity was prepared. This developmental series permitted

comparisons between age categories (three-year-old young clone and ten-year-old mature clones), sexes as well as phenotypes (normal and mantled).

Hence, for the first time, mantled reproductive development is compared alongside equivalent normal samples from the same clone, throughout the reproductive developmental process. The mantled phenotype was indistinguishable by histology till pseudocarpels were observable at the developmental category 'floral triad 3 (FT3)'.

Results revealed three novel features of mantled phenotype. Firstly, in the present set of samples, phenotypic expression of mantled was limited to pistillate flowers. Contrary to previous reports, even the abortive staminate flowers in mantled female inflorescences showed normal development while the pistillate flower of the same triad was mantled. Secondly, analysis of field sampling data revealed a lower incidence of male phase ($p<.001$) associated with the mantled phenotype. This possible effect of mantled on sex determination indicates an earlier manifestation of mantled phenotype than previously reported. Lastly, pollen samples from mantled ramets showed significantly higher pollen abortion and degeneration and lower pollen health (Chi^2 probability <0.001). Functional quality assessment of oil palm pollen grains was done through histochemical approaches and germination tests and pollen from mantled sources was analysed for the first time.

Healthy reproductive development and adequate pollination are vital for the optimal yield of oil palm. The systematic investigations undertaken here is a step towards a more comprehensive understanding of these events in normal and the mantled ramets. Results of previously uncharacterised effects of mantled phenotype call for further investigation into its phenotypic expression. Methodologies and parameters proposed here should be useful for a wide range of research into floral abnormalities of oil palm.

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LIST OF ABBREVIATIONS

%	: Percentage
%FM	: Percentage Fertility in Mantled
%HM	: Percentage of Hermaphrodite and Male Inflorescence
%M	: Mantled percentage
%SN	: Percentage Sterility in Normal
°C	: Degree Celsius
µl	: micro litre (s)
µm	: micrometre (s)
♀	: Female
♂	: Male
⚥	: Hermaphrodite
x g	: Centrifugal force
AAR	: Advanced Agriecological Research Sdn. Bhd.
AFLP	: Amplified Fragment Length Polymorphisms
AG	*: AGAMOUS*
ANOVA	: Analysis of Variance
ASF	: Abortive Staminate Flowers
AVROS	: Algemene Vereniging van Rubberplanters ter Oostkust van Sumatra
BAC	: Bacterial Artificial Chromosome
BAG	: Bcl-2 associated athanogene
bp	: base pairs
C:I	: Chloroform: Isoamyl alcohol
cm	: Centimetre (s)
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
CSIR	: Council for Scientific and Industrial Research
CTAB	: Cetyltrimethylammonium Bromide
cv	: cultivar (s)
DEF	*: DEFICIENS*
DIECA	: Diethyldithiocarbamic acid, sodium salt trihydrate
DMRs	: Differentially Methylated Regions
DNA	: Deoxyribonucleic Acid
DxP	*: Dura x Pisifera*
EDTA	: Ethylenediaminetetraacetic acid
ESTs	: Expressed Sequence Tags

F1 : Filial 1, First filial generation
FCR : Fluorochromatic Reaction
FDA : Fluorescein Diacetate
FT3 : Stage 3 Floral Triad
FT4 : Stage 4 Floral Triad
GPC : Glutaraldehyde Paraformaldehyde Caffeine Fixative
HSP : Heat Shock Protein
IUCN : International Union for Conservation of Nature
kg : kilogram (s)
kg/p/year : kg per palm per year
LFY *: LEAFY*
LINE : Long Interspersed Nuclear Elements
LSD : Least Significant Difference
M : Mantled
MF : Mature inflorescence
min : minute
mL : millilitre (s)
mM : millimolar
MPOB : the Malaysian Palm Oil Board
MRRS : Modified Reciprocal Recurrent Selection
MRS : Modified Recurrent Selection
N : Normal
ng : nano gram (s)
NGO : Non-Governmental Organisation
Nos. : Numbers
OPRI : Oil Palm Research Institute
PBS : Phosphate-Buffered Saline
PC : Pseudocarpel (s)
PC $Mean_{wt}$: Weighted mean of the number of pseudocarpels
PC $Mode_{0-8}$: Mode of the number of pseudocarpels in all fruits
PC $Mode_{1-8}$: Mode of the number of pseudocarpels in mantled fruits
PCR : Polymerase Chain Reaction
PF : 4% Paraformaldehyde Fixative
ppm : parts per million
PVP-40 : Poly Vinyl Pyrolidone-40
qPCR : quantitative Polymerase Chain Reaction
QTLs : Quantitative Trait Locus
RAPD : Random Amplified Polymorphic DNAs

REDD	: Reducing Emissions from Deforestation and Forest Degradation in Developing Countries (REDD+)
RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribonucleic Acid
RNase	: Ribonuclease
RO	: Reverse Osmosis
RSPO	: the Roundtable on Sustainable Palm Oil
SAM	: Shoot Apical Meristem
SDS-PAGE	: Sodium Dodecyl Sulphate–Poly Acrylamide Gel Electrophoresis
SIRIM	: Standards and Industrial Research Institute of Malaysia
SSR	: Simple Sequence Repeats
STI	: Stress Inducible Protein
TE	: Transposable element or Transposon
Tm	: Primer melting temperature
Tris HCl	: Tris(hydroxymethyl)aminomethane hydrochloride
TxP	: *Tenera x Pisifera*
UB	: Unripe bunch
UV	: UltraViolet
v/v	: volume/volume
w/v	: weight/volume
YB	: Young Bunch
YI	: Young Inflorescence
YT1	: Stage 1 Young Triad
YT2	: Stage 2 Young Triad

CHAPTER 1
GENERAL INTRODUCTION

The palms, of the family *Arecaceae*, are the third most economically important family of plants, after grasses and legumes. They are among the oldest cultivated trees in the world and are etched into the socio-cultural structure of many regions. Oil palm, in particular, is the central pillar of the vibrant economies of the South-East Asian countries Indonesia and Malaysia. The oil palm industry not only makes a tremendous contribution to the export revenues of these countries but also is the leading driver of socio-economic change, livelihood generation and poverty eradication in their grass-root communities (Byerlee *et al*., 2017; Rival, 2018; Qaim *et al*., 2020).

Among the major oil crops of the world, oil palm occupies the least land and produces the most abundance of oil (Mayes *et al*., 2008; Murphy, 2014; Ritchie and Roser, 2021). Palm oil and its derivatives are international commodities used for food, household, and industrial purposes. It is the single largest consumed edible oil in the world, with consumption touching approximately 70.5 million metric tons in 2017/2018 (Statista, 2019). The growing global population, increasing per capita consumption, and the rising biofuel mandates demand adequate availability of a high yielding oil crop like oil palm that offers versatility in usage (Qaim *et al*., 2020). As a consequence, global demand for palm oil is expected to double by 2050 (Corley, 2009; Pirker *et al*., 2016).

At the same time, palm oil is often deemed "environmentally un-friendly" (Ostfeld *et al*, 2019). Unsustainable expansion of oil palm plantations

through deforestation in high biodiversity areas is endangering wildlife, disrupting local livelihoods and increasingly contributing to greenhouse gas emission and thus global warming (Saswattecha *et al*., 2015, Vijay *et al*., 2016; Meijaard *et al*., 2018). This has made palm oil production and consumption highly controversial and urgent action is essential to ensure the commitment of all parties, including governments, to make palm oil production more sustainable. To this end, the UN's programme on Reducing Emissions from Deforestation and Forest Degradation in Developing Countries (REDD+) and the Roundtable on Sustainable Palm Oil (RSPO) are working with all stakeholders to develop and implement global standards for sustainable palm oil. Breeding and genetic improvements to increase productivity rather than the area under cultivation will also certainly aid in this pursuit for sustainability (Pirker *et al*., 2016).

The use of oil palm extends back to at least 5000 years in archaeological history; however, with respect to yield accomplishments and extent of scientific research oil palm remained a minor and underutilised crop till recent years (Corley and Tinker, 2008, Rival, 2017; Woittiez *et al*., 2017; Yue *et al*., 2021). Breeding and domestication of oil palm resulting in morphological and genetic changes are quite recent (Corley and Tinker, 2008). The reasons for this are two-fold. Firstly, being a heliophytic pioneer-species that thrives in land cleared and disturbed by human activities to generate high yields (Logan and D'Andrea, 2012), oil palm was able to meet the standards of a commercial crop without domestication in the traditional sense. Secondly, the nature of the plant itself (to name a few, long life cycle, large size, allogamy and heterogeneity) makes its manipulation and

improvement by natural means of plant breeding difficult (Mayes *et al*., 2008).

Oil palm breeding and selection are time-consuming, costly and exceedingly laborious. Plus, if a superior genetic material is identified, the plant offers no natural means of vegetative propagation to replicate it (Yue *et al*., 2021). Micro-propagation of oil palm through tissue culture is hence an attractive solution to the challenges involved in oil palm breeding. It helps not only to fast-track selection cycles to reach uniform planting material with reproducible high yields but also to utilise biotechnological techniques like embryo rescue of distant crosses and genetic engineering to introduce desired genetic traits and diversity. The culture of meristematic clumps in a liquid medium for the regeneration of somatic embryos was proposed in 1991 (De Touchet *et al*., 1991). The yield advantage of the clonal palms has also been documented (Wahid, 2005; Kushairi *et al*., 2010; Woittiez *et al*., 2017). However, the low efficiency of the process, as well as somaclonal variations (primarily the floral abnormality named "mantled"), still hinder its effective commercial deployment (Chan *et al*., 2014).

Over the years, oil palm tissue culture techniques have been improved and streamlined to cull out abnormalities at each stage. About 2% of high-yielding varieties of oil palm which are currently grown in South-East Asia are propagated through tissue-culture techniques that regenerate plants from young leaves. However, the oil palm farming community continues to suffer the issues of mantled palms, that is palms developing abnormal flowers that develop into fruits with reduced oil content. Young palms need several years of intensive care before they start to fruit, and it is only then

that mantled phenotype can be detected. Varying numbers of mantled palms are identified and removed in the commercial plantations at fruiting age, with a substantial loss in investments (Corley and Tinker, 2008; Weckx *et al*., 2019).

However, from a scientific perspective, the mantled abnormality has opened a new avenue for research into oil palm flower development (Low *et al*., 2017; Arraiza Ribera *et al*., 2021; Yue *et al*., 2021). The fascinating biology that occurs in flowers is boundless. Interestingly, while the underlying structure of the flower has been remarkably conserved through evolution, the number, form, and arrangement of floral organs have diversified extensively among angiosperms. Thus, the study of flower development is a major area of modern plant science. The current understanding of flower development and the complex regulatory pathways involved was greatly facilitated by research conducted on model plants such as *Arabidopsis thaliana* and *Antirrhinum majus* (Singer, 2008; Wils and Kaufmann, 2017; Dennis and Peacock, 2019).

Current technologies offer *in silico* and *in vitro* possibilities of analysing gene function. Moreover, linking genes to phenotypes through mutants is a tried and proved method in plant developmental studies. Identification and characterisation of floral-defect-mutations (especially those involving homeosis) in model species have aided in defining gene functions of a largely conserved class of genes called homeotic genes, which are involved in regulating floral organ development. For example, in the homeotic mutant *agamous* of *Arabidopsis thaliana,* stamens are transformed into petals, and carpels are replaced with a new flower. This phenotype thus

aided the identification of the function of *AGAMOUS (AG)* gene in reproductive organ (stamen and carpel) development and meristem determinacy (Uemura *et al*., 2018).

It is now known that these homeotic genes interact with one another to specify floral organ identities. However, in a tree crop like oil palm, owing to the long developmental phases and practical difficulties with mutagenesis or transformation, large-scale screening for mutants is not feasible. Naturally occurring (or in this case chance occurrence through tissue culture) floral mutants such as mantled which resembles the B class homeotic mutants in model species (Krizek and Meyerowitz, 1996), represent a golden opportunity for the scientific community to dig deeper into the reproductive pathways (Adam *et al*., 2007; Li *et al*., 2020).

Now, with the fully sequenced genome of oil palm available (Singh *et al*., 2013), the advances made in understanding the genetic mechanisms involved in flower development through the extensive research conducted on model systems and other crops could be translated to oil palm more easily than before. The 'omic' technologies enable comparative analysis of the conservation of genes and functions across species. Palm oil is derived from the flesh or mesocarp of the fruit. Understanding the intricacies of successful reproductive development in oil palm which results in the production of healthy fruit, could aid in the directed improvement of preferential characteristics and yield of cultivated palm oil (Teh *et al*., 2017).

Mantled variants, which produce fruit with little to no oil yield and cause sterility, are a problem that is decades old. Researchers have specifically

looked at the molecular and genetic aspects of the somaclonal variants and have shed light on a number of prominent differences between the normal and mantled genetic expression profile (Jaligot *et al*., 2004, 2011, 2014; Rival *et al*., 2008; Beulé *et al*., 2010). In 2015, Ong-Abdullah *et al*. found the loss of *KARMA* transposon methylation, affecting the expression of the floral homeotic gene, *DEFICIENS (EgDEF1)*, to be the underlying cause of the mantled phenotype (Ong-Abdullah *et al*., 2015, 2016, 2018; Ishak *et al*., 2020; Sarpan *et al*., 2020).

Previous studies have focussed on early detection of mantled phenotype and ascertaining the causality, to varying degrees of success (Rival *et al*., 2008, Beulé *et al*., 2010, Jaligot *et al*., 2014, Ong-Abdullah *et al*. 2015, Sarpan *et al*., 2020). Few have systematically examined the macro and micro features of the phenotype across the reproductive developmental process. The gap in previous research publications also includes standardised methodologies for characterisation of mantled fruit bunches, sampling and processing of oil palm inflorescences, and histological examination of oil palm pollen.

The present research study explores different methodologies for systematic analysis of reproductive development in normal and mantled oil palm ramets from young (3 years after planting) and mature (10 years after planting) clones. The systematic analysis here proposed is quantifiable, reproducible, and methodical, wherein measures are taken at each step to ensure reasonable comparisons. Investigation of reproductive development includes the study of the accessible range of inflorescence development stages, from early inflorescence development to fruit bunches, and

additionally the study of developing and mature oil palm pollen grains. This includes methods for

1. Determination of the genotypic and phenotypic identity of ramets.
2. Detailed characterisation of mantled fruit bunches.
3. Field sampling, sample processing and histological analysis of oil palm inflorescences.
4. Examination of morphological changes occurring during flower development in normal and mantled palms.
5. Analysis of oil palm pollen samples from normal and mantled sources.

Analysis of results is done in an attempt to better understand the reproductive development of oil palm and the abnormal and economically crippling mantled phenotype. The mantled phenotype is highly capricious and complex, and the hypothesis of this thesis is that a systematic morphological and histological analysis of oil palm reproductive development in normal and mantled ramets may reveal novel effects of the mantled phenotype.

The novelties of this study are as follows. Previous detailed investigation of oil palm reproductive development by Adam *et al* (2005) focussed on a limited number of seed-derived palms. In contrast, in the present study reproductive development of tissue culture derived ramets from a wider genetic background is examined. Further, the mantled phenotype is investigated alongside the normal, throughout the reproductive developmental process, which has not been done before. Palms are selected cautiously to avoid genotypic and environmental effects, and their genotypic

and phenotypic identities are determined for faithful comparisons within and between clones.

In past publications, the characterisation of mantled phenotype had been highly subjective (Jaligot *et al*., 2011). Here a new phenotyping and arithmetic characterisation regime for mantled is proposed which considers not only the severity but also variability of the phenotype. Phenotypic expression is examined in terms of homeotic transformation and effect on fertility. This methodical approach makes the characterisation of mantled phenotype quantifiable and reproducible.

Similarly, the field and lab protocols for effective sampling and histological analysis of oil palm inflorescence samples are explored. The strengths and limitations of the methodology for systematic analysis are discussed.

Further, the reproductive developmental stages are classified and characterised to ensure accurate comparison of developmental stages between normal and mantled, and young and mature samples. Developmental classification described here supplements the latest predictive algorithm (Sarpan *et al*., 2015) with visual staging and field references (leaf stage and length of inflorescence). Developmental series generated with a detailed description of changes during flower development allows comparison between normal and mantled samples, within and between clones of different age groups. In addition, it enables comparison to the reproductive developmental process of closely related species.

In the present study, mantled pollen samples are examined for the first time. This is done through histological techniques here standardised for oil

palm pollen. Lastly, a previously un-reported sex-specific expression of mantled phenotype, pertaining to female-specific homeosis and lower sex ratio, is explored.

In short, oil palm is the most efficient oil crop in the world; there is no doubt it is the most promising oil crop in the face of the global food crisis and climate change (Murphy, 2014; Mosnier, 2017; Rival, 2017, 2018). While advances in productivity and technology have contributed to improved food safety and efficiency of resource use, climate change and mounting pressures on natural resources are putting global food security in jeopardy. Hence, reducing hunger and poverty and improving food security in an economically, socially and environmentally sustainable manner is more vital today than ever before. Understanding this extraordinary crop is a step towards its further improvements to meet the needs of the 9 billion people who will inhabit the earth by 2050.

This study envisions creating a comprehensive knowledge base on reproductive development in oil palm species at both the structural and functional levels. Oil palm is a fascinating specimen for developmental biology studies, so this could be very valuable for the scientific community. Additionally, contrasting the normal development to that of the mantled variant is intended to untangle and streamline our understanding of the different pathways involved in the incidence of the abnormality. In turn, this could help to formulate strategies for the improvement of the tissue culture and breeding process to attain sustainability in oil palm cultivation. The socio-economic implications of this could be paramount.

1.1. GENERAL OBJECTIVES

The objectives of the current research, as explored in the different chapters, are as follows:

- Identification of comparable normal and mantled ramets belonging to different clones and molecular and morphological characterisation of ramets for accurate determination of genotypic and phenotypic identity (Chapter 3).
- Detailed phenotypic characterisation of mantled fruit bunches to account for the heterogeneity in homeotic transformation and fertility associated with the phenotype (Chapter 4).
- Optimisation of inflorescence sampling protocols for the non-destructive and destructive sampling range, including evaluation of the efficacy of inflorescence length as a field reference for the inflorescence stage (Chapter 5).
- Comparison of different fixatives available to optimise microscopy protocol for oil palm inflorescence samples (Chapter 5).
- Developmental classification of collected samples to identify comparable normal and mantled samples from each sampling group, and detailed characterisation of oil palm inflorescence developmental stages for easy prediction based on field observations and histology (Chapter 6).
- Histological analysis of oil palm inflorescence samples to construct a detailed description of the developmental stages in the form of a reproductive developmental series and to compare normal and mantled reproductive development (Chapter 7).

- Establishment of protocols for assessment of the functional quality of oil palm pollen and comparison of the functional quality of pollen samples from normal and mantled sources (Chapter 8).
- Comparison of the number of male inflorescences produced by normal and mantled clones to examine the possible effect of mantled on sex determination (Chapter 9).

CHAPTER 2
BACKGROUND

2.1. PALM OIL PROSPECTS AND CHALLENGES

Oil palms (*Elaeis spp.)* are perennial palm species that grow best within 10 degrees of the equator. The genus *Elaeis* primarily consists of two species namely *Elaeis guineensis* and *Elaeis oleifera*, which are presumed to have drifted apart during the prehistoric separation of the African and American continents. They are commercially cultivated for the extraction of oils. *E. guineensis* endemic to tropical Africa has a higher yield and is widely cultivated in South-East Asia whereas *E. oleifera* from Central and South America has higher unsaturated fatty acid content, shorter stature and superior resistance to diseases and hence has significance in the breeding of the crop (Singh *et al*., 2013). In this thesis, the term oil palm is used to refer to *E. guineensis* unless otherwise specified.

The oil from oil palm is derived from either the fruit mesocarp (20% oil) or the seed kernels (3% oil), referred to as palm oil and palm kernel oil, respectively (Soh *et al*., 2003). Palm oil is versatile and is the raw material for an extensive product chain. Crude palm oil can be separated into a wide range of distinct oils with different properties, and hence can replace animal fat and other vegetable oils in a wide variety of products (Lai *et al*., 2015). In the crude form, it is used as a source of carotene for the production of Vitamin A supplements and natural dyes for snack food (Corley and Tinker, 2008; Loganathan *et al*., 2017; Martianto *et al*., 2018).

After refining, it is used as cooking oil, salad oil, and as an ingredient for most margarines, confectionaries, and ice-cream. Refined palm oil is also used as the base for most liquid detergents, soaps, shampoos, lipstick, waxes, and polishes (Keng *et al*., 2009). Its potential to produce biofuel is also actively explored today (Sang 2003; Kurnia *et al*., 2016; Jin *et al*., 2021). Palm oil is also used for the production of fatty acids and alcohols after fractionation (Kellens *et al*., 2007). The palm kernel oil is chiefly used in the food and oleo-chemical industries, and the residue of kernel oil extraction namely palm kernel cake is a valuable protein-rich animal feed (Alimon, 2004; Rupilius and Ahmad, 2007; Corley and Tinker, 2008).

Oil palm is a highly efficient oil producer, and so it requires ten times less land than other oil-producing crops to produce the same amount of oil (Figure 2.1; Mayes *et al*., 2008; Murphy, 2014; FAO, 2021; USDA, 2021). Thus, palm oil is produced from roughly 5% of the total global land area used for vegetable oil production and accounts for 33% of vegetable oil and 45% of edible oil worldwide (Singh *et al*., 2013). The world devotes over 300 million hectares of land for oil crop production. In terms of productivity, that is the amount of oil produced from a unit area of land, oil palm far surpasses the alternatives. From each hectare of land, you can produce about 3.92 tonnes of palm oil, that is around five times higher than alternatives such as sunflower or rapeseed oil (productivity of 0.74 t/ha); and 14 to 19 times higher than popular alternatives such as coconut or groundnut oil (productivity of 0.29 t/ha and 0.21 t/ha respectively; Figure 2.1; FAO, 2021).

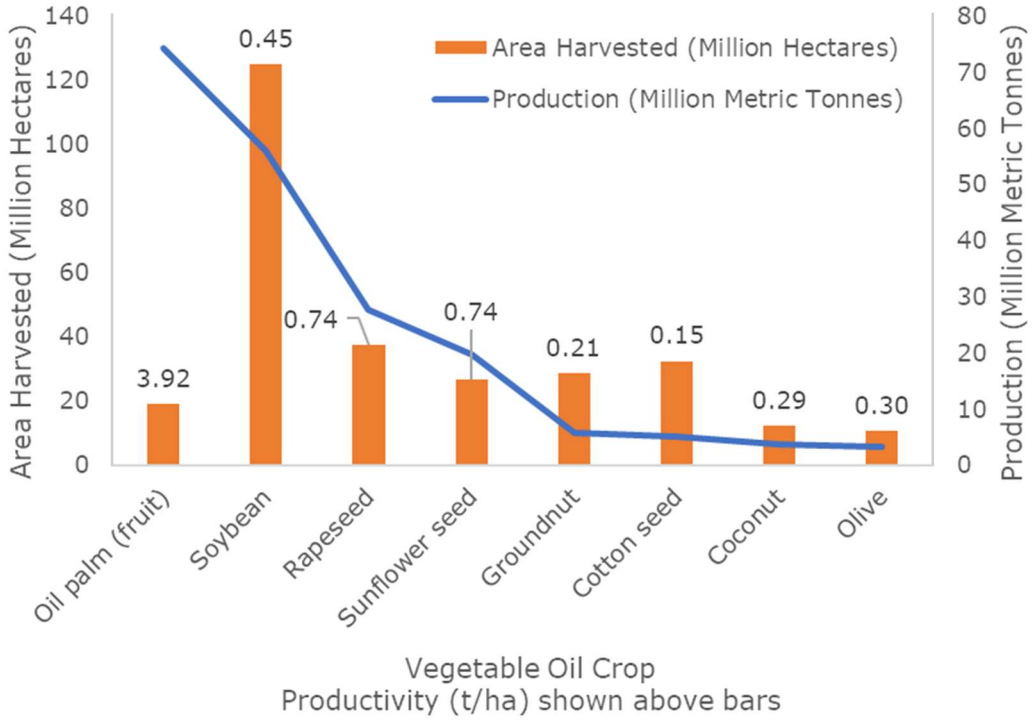

Figure 2.1 Global Area Harvested, Production and Productivity of Major Oil Crops for the Year 2018. The global area harvested (FAO, 2021) is in million hectares (y-axis) and is shown as a bar graph. The global production of different vegetable oils (USDA, 2021, p-10) is in million metric tonnes (secondary axis) and is shown as a line graph. The calculated productivity in tonnes per hectare of each vegetable oil crop is displayed as data labels on top of the bars.

Global production of oils and fats, in general, has grown by over 50% over the last decade (Figure 2.2). Global vegetable oil production increased ten-fold since the 1960s – from 17 to 170 million tonnes in 2014. This mainly consisted of the increase in production of the major oils, derived from palm, soybean, canola, and sunflower seed which together account for 70% of the global production. Production of palm oil has grown faster than any other vegetable oil in direct response to the changes in world demand. From being one of the minor oils being produced and consumed in 1976 (1.6% and 6% of the world production and consumption of oils and fats, respectively) palm oil gradually surged to become the highest produced and consumed oil in 2005, overtaking soybean (Figure 2.2; MPOB, 2011). The major exporters of palm oil are Indonesia and Malaysia, and the top importer is China, closely followed by India (FAO, 2021).

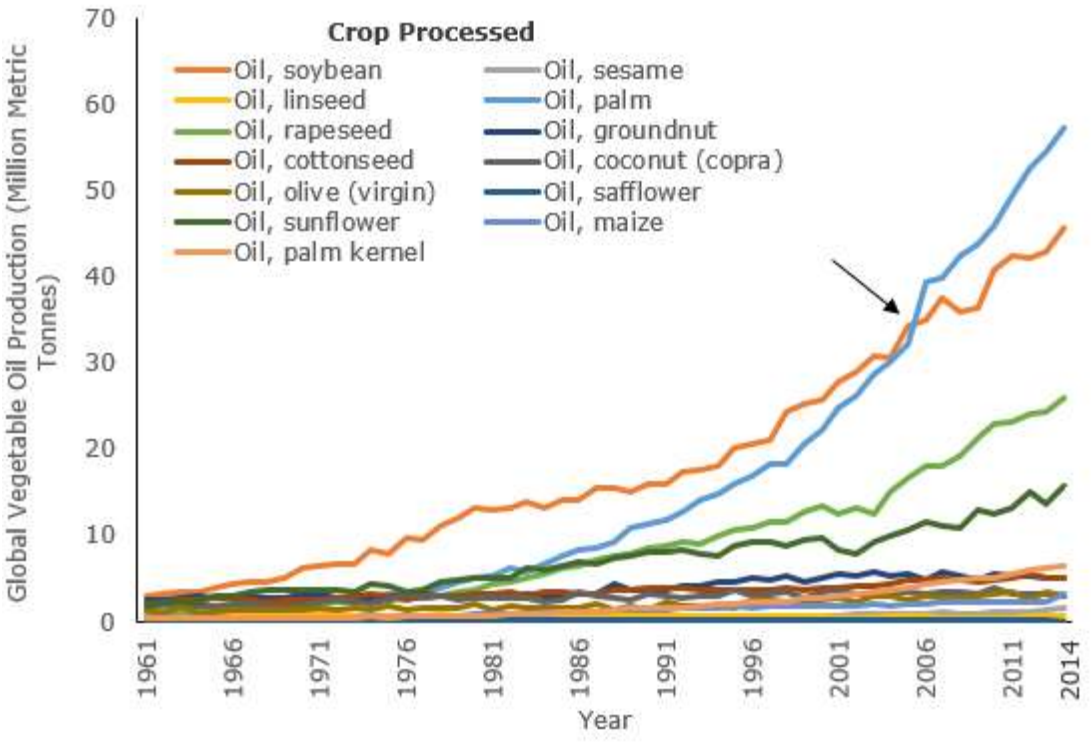

Figure 2.2 The Change in Global Production of Vegetable Oils from 1961 to 2014. The global vegetable oil production (FAO, 2020) is in million metric tonnes (y-axis). Each coloured line graph represents a different source of vegetable oil as indicated by the index key. Arrow shows palm oil production overtaking soybean oil in 2005 to become the highest produced vegetable oil in the world.

This rapid expansion of palm oil production has promoted economic growth in producing countries but at the same time has resulted in the clearing of substantial tracts of tropical forests to make room for the large plantations (Qaim *et al*., 2020). Unsustainable expansion of oil palm mono-cropping plantations through deforestation in high biodiversity areas is endangering wildlife such as orangutans, elephants, and tigers, disrupting local livelihoods and dramatically increasing the release of greenhouse gases that contribute to global warming (Oosterveer, 2014; WWF, 2013; Tapia *et al*., 2021). This has made palm oil production and consumption highly controversial today.

Online activist groups, including Greenpeace, had campaigned to boycott palm oil citing the industry's role in deforestation and habitat degradation in the countries where it is produced. Yet, banning palm oil would not end

biodiversity loss, according to a 2018 report by the International Union for Conservation of Nature (IUCN); it would only increase the production of more land-hungry oil crops to meet the demand for oil thus displacing and even worsening the global biodiversity losses (IUCN, 2018). Substituting palm oil with other oil crops will only accelerate deforestation since oil palm is biologically far superior to other oil crops in-terms of efficiency in land use and productivity (Figure 2.1). Furthermore, unlike the other oil crops, oil palm is perennial with twenty-five years of economic life cycle; therefore, it does not require yearly replanting and yields fresh fruit bunches all year long. This conserves energy and reduces soil erosion resulting from continued tillage (Murphy, 2014; Woittiez *et al*., 2017).

Oil palm is the central pillar of Indonesian and Malaysian economies, by way of its contribution to the export revenue and also to the number of livelihoods generated in the production, processing and trading sectors (Sheil *et al*., 2009; Sayer *et al*., 2012). A ban or boycott will uproot the livelihoods of millions. A ban will also reduce any incentives for producers to adopt sustainable production practices. There is no simple solution for the disastrous impact of palm oil on biodiversity. Urgent action is necessary to ensure the commitment of all parties, including governments, to make palm oil production more sustainable (Koh and Wilcove, 2007; Fitzherbert *et al*., 2008; Khatun *et al*., 2017; Rival, 2017; 2018).

For this reason, palm oil plantations are now being certified according to the Roundtable on Sustainable Palm Oil (RSPO). RSPO unites stakeholders from oil palm producers, processors or traders, consumer goods manufacturers, retailers, banks/investors, and environmental and social non-governmental

organisations (NGOs), to develop and implement global standards for sustainable palm oil. Malaysia and Indonesia are making it mandatory to have all palm oil plantations, including smallholders, to be certified according to national standards over the coming decade.

Other concerns surrounding palm oil such as health effects of the highly consumed oil (Mukherjee and Mitra 2009; Fattore and Fanelli, 2013; Mancini *et al*., 2015) and impact on local communities and indigenous people of South-East Asia (Colchester *et al*., 2006; Dallinger *et al*., 2011; Hall, 2011; Qaim *et al*., 2020) are also highly debated and are currently being addressed by the scientific community.

From a plant-breeding point of view, attaining the yield potential of this remarkably productive crop will help to attain increased productivity rather than the area under cultivation. Therefore, achieve sustainability for biofuels and edible oils without increase in the rainforest footprint. Favourably the estimated potential yield of oil palm is 18 t ha^{-1}, of which only less than a quarter is currently achieved in the field (Barcelos *et al*., 2015; Tapia *et al*., 2021).

The present study addresses broader scientific questions related to reproductive development of oil palm in an attempt to expand our understanding of normal and abnormal (mantled) floral physiology. It is hoped that the data generated through this research will help in future endeavours to improve this exceptional crop to meet the oil demands of the future. In this chapter the current knowledge base is explored in terms of practices and challenges involved in oil palm breeding (Section 2.2), the structural and functional aspects of oil palm reproduction (Section 2.3), and

aspects of the abnormal phenotype under investigation, namely mantled (Section 2.4).

2.2. OIL PALM BREEDING

Optimisation of palm oil production by genetic improvement of the plant material has attracted attention due to the increasing commercial interest in oil palm ever since the late 1970s. Oil palm is an open-pollinated, highly heterozygous perennial tree crop. The long generation time, large amount of land and resources required for valid experimental design and strenuousness of controlled pollination causes oil palm breeding by conventional methods, that are successful in annual oil crops, to be very slow, labour intensive and expensive (Jaligot *et al*., 2011; Mayes *et al*., 2008).

2.2.1. Hybridisation and Selection

The current commercial oil palm planting materials are mostly *Dura* x *Pisifera* (DxP) hybrids, otherwise known as *Tenera* (T) hybrids, bred following either the Modified Recurrent Selection (MRS; Figure 2.3) or Modified Reciprocal Recurrent Selection (MRRS; Figure 2.4) or a combination of the two. In MRS the *Pisiferas* selected by DxP test and the *Duras* selected for their performance are used to produce commercial DxP seeds (Figure 2.3). In MRRS selfed *Duras* and the selfed *Pisiferas* are crossed to produce the commercial DxP seeds (Figure 2.4). When these two methods, MRS and MRRS, are combined, the genetic variability of commercial DxP could be further reduced (Tan *et al*., 2013).

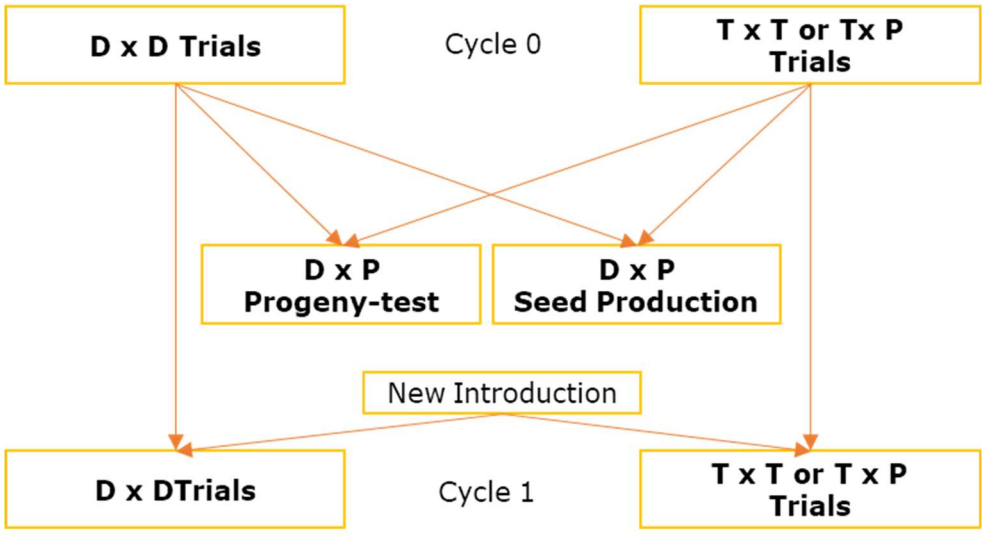

Figure 2.3 Schematics of Modified Recurrent Selection (MRS) in Oil Palm Breeding. D, P and T stands for *Dura*, *Pisifera* and *Tenera* respectively. Selected *Duras* and *Pisiferas* are used to produce commercial DxP (*Tenera*) seeds. *Dura* and *Tenera* selections are done based on individual, family and progeny test performance. *Pisiferas* are selected based on *Tenera* sib and progeny test performance. Adapted from Rajanaidu *et al*. (2000).

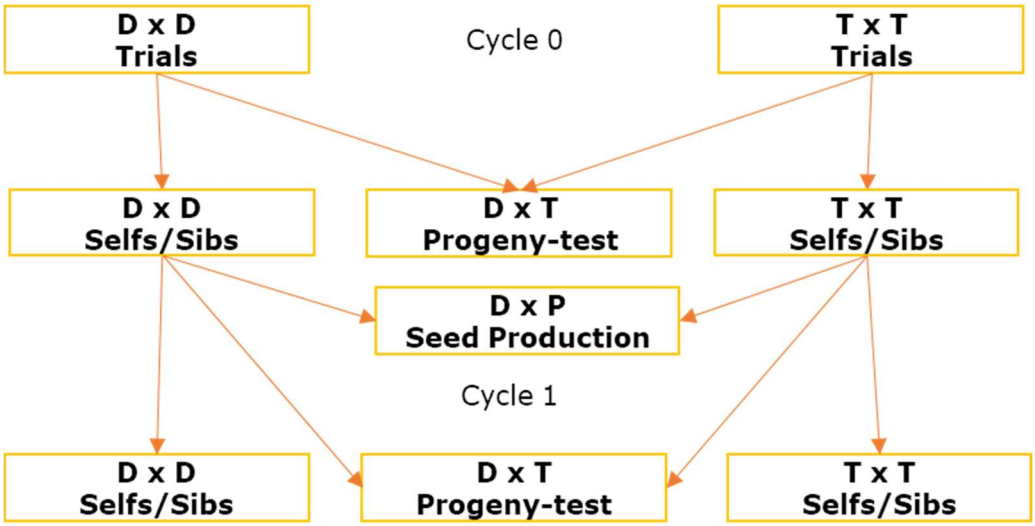

Figure 2.4 Schematics of Modified Reciprocal Recurrent Selection (MRRS) in Oil Palm Breeding. D, P and T stands for *Dura, Pisifera* and *Tenera* respectively. Selfed *Duras* and the selfed *Pisiferas* are crossed to produce the commercial DxP (*Tenera*) seeds. Selection is based on progeny test performance. Adapted from Rajanaidu *et al*. (2000).

However, the commercial DxPs produced are not F1 hybrids and they are not homogenous within families. The production of these commercial hybrid seeds of acceptable genetic homogeneity requires selection cycles lasting roughly ten years. Besides, currently, the breeding populations exhibit lower genetic variability and low heritability for the oil yield (Tan *et al*., 2013, Soh *et al*., 2011).

2.2.2. Clonal Propagation

Oil palm does not have means of natural vegetative propagation (such as offshoot propagation). Asexual propagation through conventional horticulture (such as cutting, bud grafting, and suckers) is also not possible in oil palm. Hence micropropagation by tissue culture is the only resort for mass production of high yielding elite genetic material, in this crop. Tissue culture also offers an opportunity for early commercial exploitation of new genetic materials from wide crosses and introgression programs. This will broaden the genetic base of the commercial plantings, thus reducing the risk of genetic vulnerability to biotic and abiotic stresses, and also ensuring sustainability.

Semi-clonal seed approach is used by breeders to reproduce best tested DxP families in bulk. Tissue culture is used to multiply the parental *Dura* and thus produced clonal mother palms are then pollinated with the tested *Pisifera*, to produce semi-clonal seeds, that is seeds produced by having one ramet parent and a sexual parent. They offer 15% yield gain over conventional DxP hybrid seeds and greater degree of uniformity because the crossings are confined to a limited number of parental combinations. Cost of seed production and risk of clonal abnormality is very much lower in semi-clonal seeds compared to tissue culture plantlets (Kushairi and Amiruddin, 2020).

Another synergetic area of research has been the production of synthetic seeds using matured somatic embryos that offer amenability to long term storage and low-cost delivery (Mariani *et al*., 2014; Karim, 2021). Further, tissue culture opens avenues for the creation of novel genetic variants by

modern genetic techniques such as genetic transformation and genome editing via CRISPR/Cas 9 system (Bortesi and Fischer, 2015; Yarra *et al*., 2019; Yeap *et al*., 2021).

The tissue culture technique for oil palm developed in the 1970s was quickly capitalized and commercialized by the oil palm industry, in the interest of replicating superior genotypes (Soh *et al*., 2001, 2011). *Tenera* cloning is performed from the selected individuals (ortet) that were superior among the DxPs. The prevalent starting explant for oil palm tissue culture is the spear leaf. The explant in callus induction media produces nodular callus along the cut edges. Some of the calli undergo embryogenesis forming somatic embryos that generate shoots and roots under the right conditions resulting in viable plantlets, called ramets (Tan *et al*., 2013).

Accurate ortet selection and field level performance testing of the clones ensures genetic improvement via clonal propagation. Accordingly, the field trials with clonal material showed encouraging yield improvement owing to the reproducibility of superior genetic potential and uniformity exhibited by them. Soh *et al*. (2011) reported 18% higher oil yield of clonal material as compared to DxP. Tan *et al*. (2013) noted that the minimum oil yield standards of DxP *Tenera* hybrids and *Tenera* clones defined by Standards and Industrial Research Institute of Malaysia (SIRIM) are 42.5 kg/p/year and 55 kg/p/year respectively against the 18 kg/p/year yield of early planting materials *Deli Dura*. Kushairi *et al*. (2010) estimated the annual market demand for oil palm tissue culture plantlets to be more than 100 million, which is double the current global production.

2.2.3. Limitations of Oil Palm Tissue Culture

Despite the availability of established means of micro-propagation for oil palm, inefficiencies of the tissue culture technique in terms of low amenability, problems in clonal fidelity and abnormalities associated with somaclonal variations hindered the expansion of this industry. Oil palm is a recalcitrant plant, that is oil palm cells, tissues and organs do not respond to tissue culture manipulations as easily as other plant species. The rates of callogenesis and somatic embryogenesis in oil palm tissue culture are around 19% and 3-6% respectively and remain the major bottlenecks (Low *et al*., 2008). In addition, somaclonal variations are a prominent threat to oil palm tissue culture since the abnormal phenotypes like mantled causes a huge waste of investments by escaping early detection (See section 2.4; Jaligot *et al*., 2011). Hence, presently around 98% of commercial oil palm planting material consists of hybrid *Tenera* seeds and only the remaining 2% is tissue culture derived plantlets (Kushairi *et al*., 2010; Weckx *et al*., 2019).

All plant materials used in the current study were tissue culture derived *Tenera* ramets. The clones were selected to account for the major breeding populations of oil palm (Chapter 3). A closer examination of the deleterious somaclonal variation, mantled, that results in floral abnormalities is undertaken here (Chapter 4). The high incidence of mantled abnormality is one of the major factors that currently hinder effective deployment of clonal progeny. Here, ramets of normal and mantled phenotype were carefully selected for comparison of inflorescence development (Chapter 7) and pollen functional quality (Chapter 8). Further, effect of mantled phenotype

on sex determination is also examined (Chapter 9). A review of the mantled somaclonal variant based on previous literature and our current understanding of the molecular basis of the origins of this phenotype is expanded in the section 2.4.

2.2.4. Bridging the Gap with Biotechnological Tools

Researchers are more and more aware of the gap between the progress in breeding and biotechnology and are actively trying to bridge it. Oil palm is a diploid species with 16 pairs of chromosomes (2n=32). Many biotechnological resources are now available in oil palm such as Bacterial Artificial Chromosome (BAC) libraries, methylation filtration sequence, Expressed Sequence Tags (ESTs), microarrays, microsatellite maps and the fully sequenced genome (Mayes *et al*., 1997; Billotte *et al*., 2005; Singh *et al*., 2013; Low *et al*., 2017).

Analysis of genetic diversity and population structure, identification of trait-associated markers and genotype characterization have been possible in oil palm using molecular markers such as Amplified Fragment Length Polymorphisms (AFLPs), Random Amplified Polymorphic DNAs (RAPDs) and Simple Sequence Repeats (SSRs) (Billotte *et al*., 2005). The first genetic map of oil palm was constructed using Restriction Fragment Length Polymorphism (RFLP) markers (Mayes *et al*., 1997), which resulted in mapping monogenic trait SHELL, responsible for the different fruit forms in oil palm (*Dura, Pisifera* and *Tenera*).

RAPD markers were the most popular PCR-based markers initially, but their application was not very successful due to problems with reproducibility. The Malaysian Palm Oil Board (MPOB) was more successful with AFLPs which

provided the means to generate huge numbers of dominant markers without any prior sequence information. The limitation was its dominant nature; however, AFLP revealed clear Mendelian inheritance and was adopted for map saturation. Genomics-based microsatellites or SSR markers were initially reported for oil palm by Billotte *et al*. (2001). They are highly variable, co-dominant, chromosome-specific and easy to use.

Initial genetic maps have now been expanded to construct comprehensive genetic maps. They have since provided a useful means of determining Quantitative Trait Locus (QTL). QTLs for shell thickness, yield components, fatty acid composition of palm oil and stem height have been studied in oil palm (Rance *et al*., 2001; Montoya *et al*., 2013; Lee *et al*., 2015). Linkage maps can greatly accelerate future breeding programs.

ESTs have become the tool of choice for rapid gene discovery. The first oil palm EST paper was published by Jouannic *et al*. in 2005, studying genes expressed in inflorescences, shoot apices and zygotic embryos of normal and mantled clones. ESTs, in turn, led to the development of microarrays for gene expression studies. With the exponential increase in genetic data generated, the technology to support and store the data also developed, the PalmGenes database is an example (Low *et al*., 2017).

Molecular breeding programs using trait-associated molecular markers can significantly impact the rate at which genetic improvement can be achieved (Babu *et al*., 2019). The importance of the same was noted by Tranbarger *et al*. (2012) while describing SSR markers in transcripts (EST-SSRs) involved in vegetative and reproductive development of oil palm. Congruently, Xiao *et al*. (2014) endeavoured to identify EST-SSRs involved

in cold stress response which can potentially serve as markers for cold tolerance in oil palm.

In 2013, the oil palm genome of 1.8 Gb size was reported by Singh *et al*. (2013), further speeding up identification of markers and the application of genomic tools in oil palm breeding (Ong *et al*., 2020). Bhagya *et al*., (2020) characterised 150 oil palm genotypes with respect to 12 quantitative variables using 54 genomic microsatellite markers. In 2021, Gan *et al*., used molecular markers for the assessment of genetic diversity and population structure of oil palm field genebank (Gan *et al*., 2021).

Genetic transformation studies have also been carried out in oil palm. Lipid composition would be a potential target for engineering via transgenic approach (Parveez *et al*., 2000, Abdullah *et al*., 2005). Immature embryos have been regarded as a promising starting material for both direct and *Agrobacterium*-mediated gene transfer. In 2003, stable integration of insect resistance gene in transgenic oil palm was reported (Abdullah *et al*., 2003). Another interesting research in this area has been on the feasibility of manipulating the lignin pathway so as to create a tolerance to *Ganoderma* rots which is a serious problem in oil palm (Paterson *et al*., 2009).

Biotechnological and bioinformatics tools are transforming the face of oil palm breeding and germplasm conservation, like any other crop. At present, study of global patterns of gene expression is possible via microarrays and transcriptome sequencing. Transcriptome-based microarray with 105,000-probes from oil palm mesocarp was reported by Wong *et al* (2014). Undoubtedly further improvements of breeding and selection of superior oil palm planting material will be by genomic selection of key yield traits (Cros

et al., 2015; Wong and Bernardo, 2008). Traditional breeding techniques, coupled with biotechnology, are currently being used in efforts to tackle stagnating yields, control diseases, improve oil quality, and increase versatility and adaptability to climate change (Soh *et al*., 2017; Ong *et al*., 2020).

In the present study, previous literature on biotechnological and bioinformatic research was used to put physiological observations in the context of underlying genetics. Moreover, molecular methods (SSRs and *KARMA* assay) were employed to determine the genotypic and phenotypic identity of ramets (Chapter 3).

2.3. BOTANY OF OIL PALM

Oil palm is an evergreen tropical perennial, sometimes attaining more than 100 years in age (Corley and Tinker, 2008). This tree-like monocot (Figure 2.5 A) reaches 12 to 15m in height and approximately 45cm in diameter at the end of its economic lifespan (20-30 of age).

A single vegetative shoot apical meristem (SAM) initiates the entire above ground structure of the plant. The SAM is localized in a basin like depression within a soft mass of young leaves at the crown of the palm and is maintained throughout the lifetime of the plant. In two to three years from initiation, fully developed leaflets unfold in the centre of the palm crown.

Figure 2.5 Botanical Illustration of the Oil Palm by Franz Eugen Köhler (1887). The illustration contains A) a whole palm, B) a male inflorescence, C) a male spikelet, D) a female spikelet, E) a female inflorescence/ developing fruit bunch, 1) a developing flower/ flower bud, 2) a male flower at anthesis, 3) longitudinal section of a male flower showing aborted pistilode and anthers, 4) anthers and connate filaments of a single male flower, 5) a female flower at anthesis, 6) longitudinal section of a female flower, 7) cross section of ovaries, 8) a developing fruit, 9) a fruit after the upper half of the pulp is removed showing the shell, 10) cross section of a fruit with a single kernel, 11) longitudinal section of a fruit with two kernels, 12) cross section of a fruit with two kernels, 13) an oil palm kernel or seed, 14) cross section of a single kernel and 15) longitudinal section of a seed kernel showing the embryo. Source: Greenpeace (2012).

Under favourable climatic conditions, a new leaf primordium is produced approximately in every two weeks or more frequently as in the case of young palms. Mature leaves also known as fronds could be 8 meters in length (Figure 2.5; Adam *et al*., 2005; Corley and Tinker, 2008; Legros *et al*., 2009a; Jouannic *et al*., 2011; Forero *et al*., 2012).

2.3.1. Morphology of Oil Palm Inflorescences

In oil palm male and female flowers are produced in separate inflorescences which are botanically compound spadices (Figure 2.5 B, E). In a spadix, small flowers are borne on a fleshy stem, typically surrounded by a leaf like bract called a spathe (Singer, 2008). In a compound spadix like in oil palm, the inflorescence is branched. The central rachis gives rise to branches known as rachillae or spikelets on which functionally unisexual sessile flowers are borne (Figure 2.6; Adam *et al*., 2005).

Oil palm exhibits temporal dioecy, where the same palm goes through separate female and male phases in their life cycle. The different phases vary in their duration depending on the genetics, age and particularly environmental conditions. Occasionally at the transition between them hermaphrodite inflorescences (Figure 2.6 B) are produced (Rival, 2007).

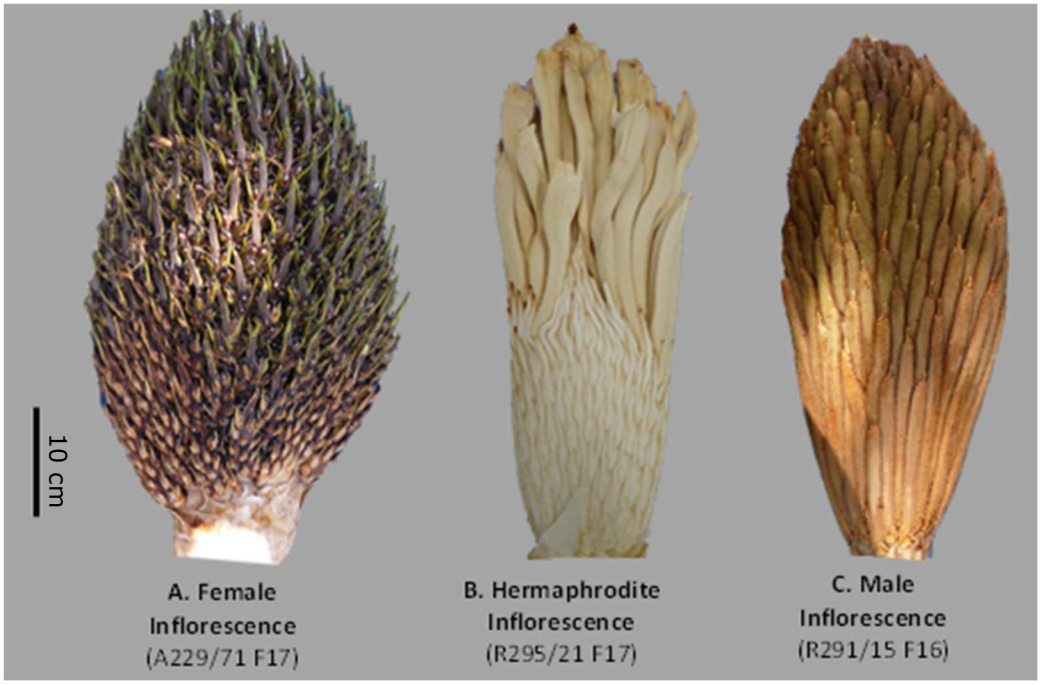

Figure 2.6 Different Sexes of Oil Palm Inflorescences. Figure shows from the left a female (A), a hermaphrodite (B) and a male (C) inflorescence of oil palm, from the sampling range. Sample IDs are included within brackets in the format Clone/Accession number and leaf stage. Accession number indicates the sampled palm, leaf stage in the format F## indicates the arrangement of the frond from which the inflorescence was extracted.

The inflorescences develop highly protected deep within the crown of the palm. Developing inflorescences are protected by two spathes (prophyll and peduncular bract; figure 2.7 A) and the subtending leaf base. The prophyll and the peduncular bract tightly enclose the expanding inflorescence until approximately six weeks before flower maturity (Corley and Tinker, 2008). The peduncle, or the stalk of the inflorescence (Figure 2.7 A, B) reaches a length approximately 20–30 cm in female and around 40 cm of length in male, at maturity (Adam *et al*, 2005).

The spikelets are arranged spirally around the central rachis: 100–300 in male (Figure 2.8 A, B) and approximately 150 in female (Figure 2.6 A, 2.7). The male inflorescence contains spikelets, each bearing 400–1500 single functional staminate flowers subtended by floral bracts (Figure 2.8 C; Adam *et al*, 2005; Corley and Tinker, 2008).

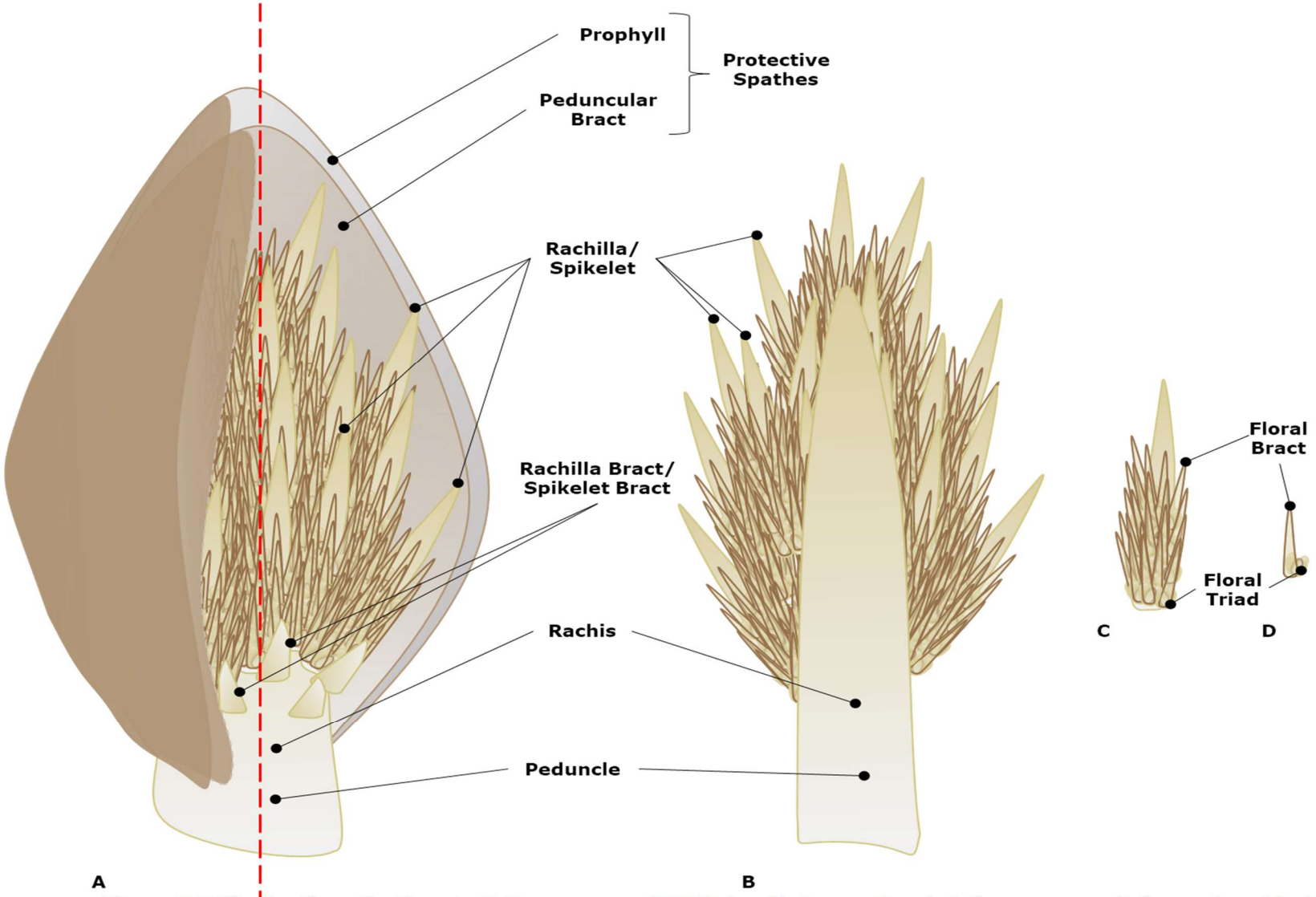

Figure 2.7 Illustration of a Female Inflorescence of Oil Palm. A) shows a female inflorescence partially enveloped by the protective spathes, B) shows the longitudinal section of the same, relative position is indicated by the dotted red line in A, C) depicts the structure of an individual spikelet and D) an individual floral triad. Figures are labelled to show the different parts (Original Illustration).

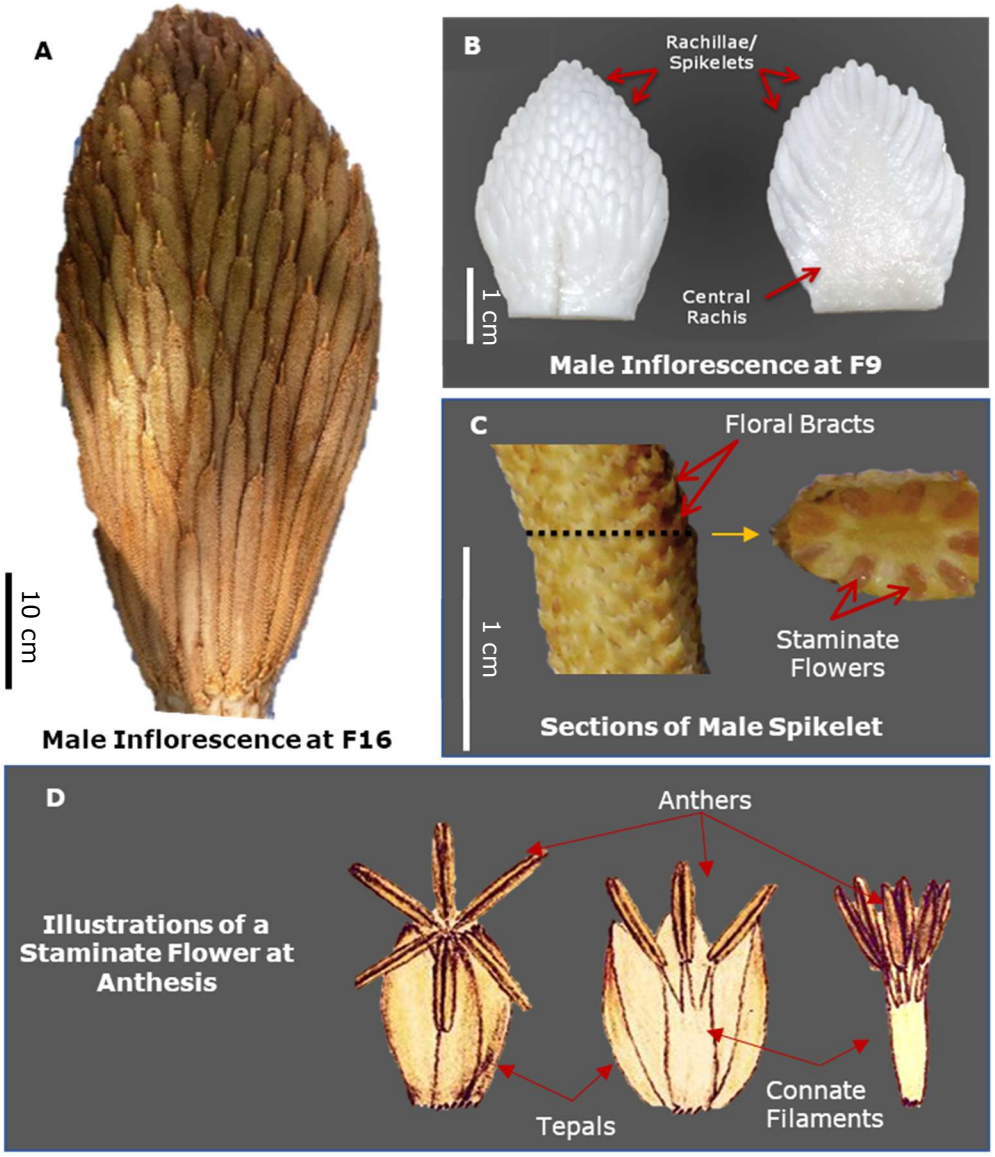

Figure 2.8 Structures of Male Inflorescence of Oil Palm. (A) photograph of a male inflorescence prior to anthesis from leaf stage F16. The spikelets are arranged spirally around the central rachis. (B) Photographs of immature male inflorescence from leaf stage F09 and its longitudinal section, showing arrangement of rachillae/spikelets on the central rachis. (C) Photographs of sections of a male spikelet. Frontal view shows floral bracts. Individual flowers are enclosed within these floral bracts. The cross section shows arrangement of staminate flowers. The relative position of the cross section is indicated by the dotted line and arrow. (D) Illustrations of the structure of a staminate flower at anthesis. Pictures depict from the left an individual flower with protruding anthers, position of androecium within the whorl of tepals and anthers and connate filaments of a single male flower (Original Illustration)

In female inflorescence (Figures 2.6 A, 2.7) each spikelet bears 5–30 floral triads (Figures 2.7 D, 2.9), consisting of single pistillate flowers flanked by two abortive staminate flowers (pedicellate) subtended by a bigger spiny floral triad bract (Figure 2.9). This is suggestive of an ancestral hermaphrodite inflorescence with functional floral triads. Flowers destined to be male or female later undergo a programmed degeneration of their gynoecium or androecium early in reproductive development. The "progression from bisexual to unisexual flowers and from monoecy to dioecy" has been pointed out by Moore and Uhl (1982), as a general trend in the evolution of floral characters within the palm family (Adam *et al*., 2005, de Farias *et al*., 2018).

In oil palm, the individual flowers are trimerous, that is having floral organs arranged in groups of three, as is typical of monocotyledoneous species flowers. Individual flowers consist of an outer floral bract (or bracteole in the case of flowers of the triad), followed by perianth organs (Figure 2.9). The perianth is composed of 3 sepals (calyx) and 3 petals (corolla) and surrounds the reproductive whorls, the androecium, and the gynoecium. The sepals and petals are similar in appearance and are often referred to as tepals (Figure 2.9; Corley and Tinker, 2008).

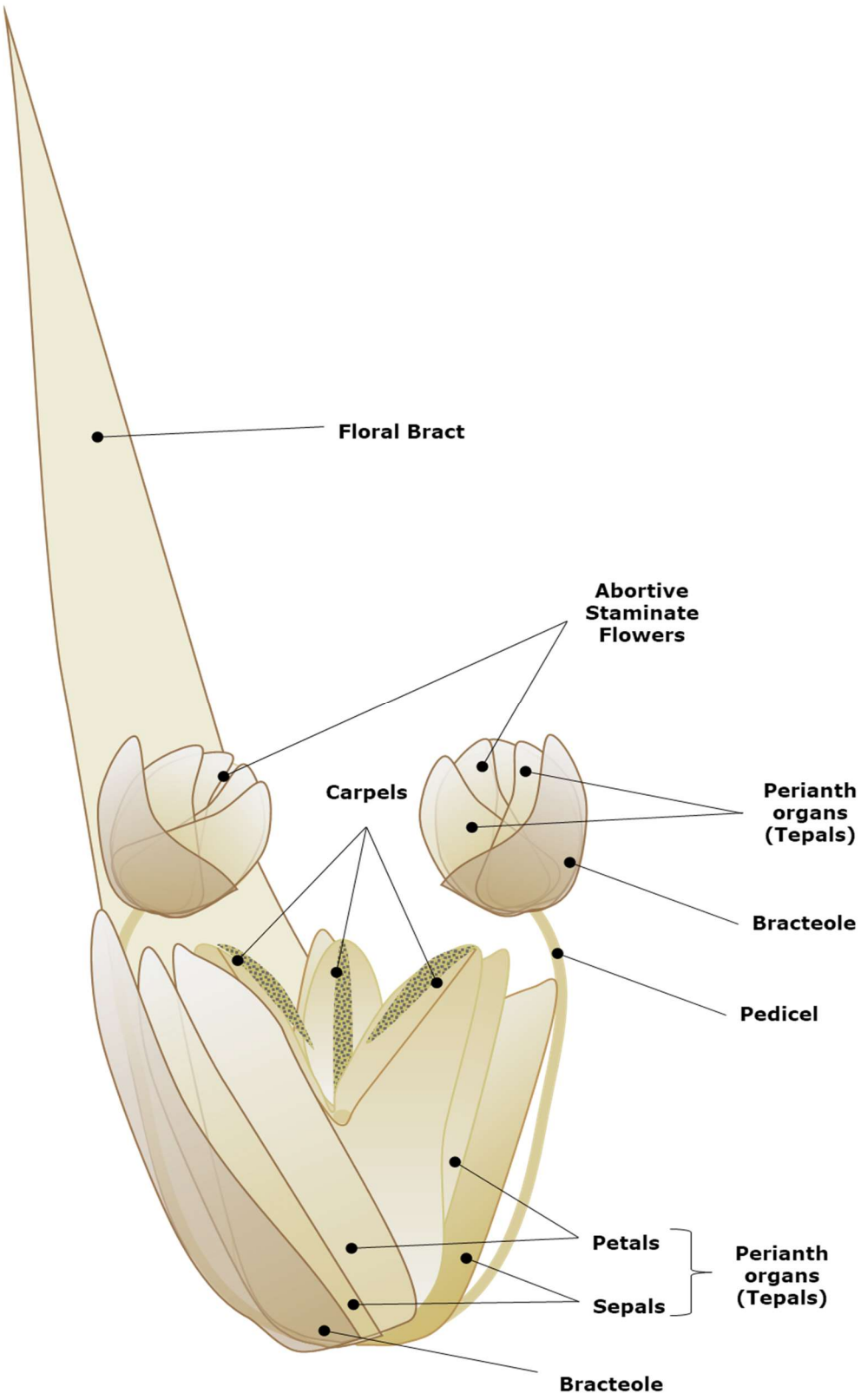

Figure 2.9 Illustration of a Floral Triad from a Female Inflorescence. The floral triad consists of a functional pistillate flower flanked on either side by abortive staminate flowers (ASFs). The ASFs are pedicellate while the pistillate flower is sessile. All three flowers of the triad have perianth organs (sepals and petals) and a bracteole surrounding the reproductive whorls. The reproductive whorls of the pistillate flower are composed of 6 staminodes (not shown) and 3 carpels (Original Illustration).

The androecium of staminate flowers consists of stamens with connate filaments arranged in two concentric whorls and bilobed anthers. The gynoecium of pistillate flowers consist of three carpels that are composed of 3 stigmatic lobes and fused styles and ovaries (Figure 2.9). Though flowers are unisexual, the early development of the male and female flowers is identical. The stamen and carpel primordia are initiated in both but one of them is arrested later on in development to form unisexual flowers (Adam *et al*., 2005; Corley and Tinker 2008). Adam *et al*. (2005) observed this divergence at carpel initiation. Thus, the pistillate flowers have rudimentary stamens (staminodes) surrounding the three fused carpels and the staminate flowers have an abortive pistillode in the middle of the six stamens. (Figure 2.10).

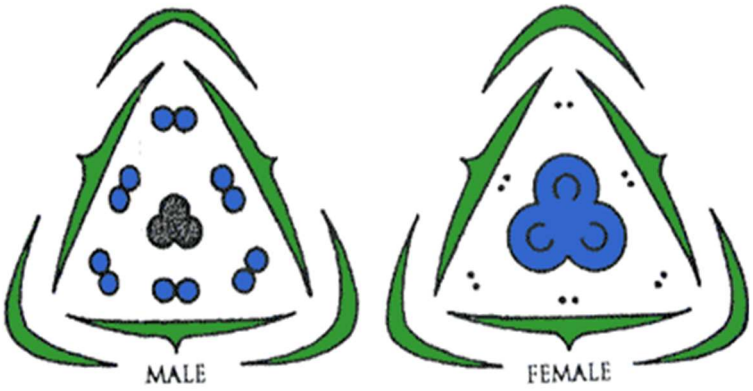

Figure 2.10 Floral Diagram of family *Arecaceae*. Figure shows outer two whorls of tepals (green) in both sexes, six stamens of connate filaments (blue) surrounding a pistillode in male flower and tricarpellate gynoecium (blue) surrounded by six staminodes (black) in female flower. Source: thewildclassroom (2014).

2.3.2. Reproductive Development in Oil Palm

Study of flower development is a major research focus predominantly due to the obvious economic significance of flowers and the fruits and seeds they produce. Development of the flower is governed by complex regulatory mechanisms at genome and epigenome levels, in response to the various environmental and endogenous cues, a wide range of which is reported to be conserved among flowering plants. Significant progress has been made in understanding the underlying molecular mechanisms of flower development in the last couple of decades (Krizek and Fletcher, 2005; Wellmer *et al*., 2006; Zhang and Wilson, 2009; Irish, 2010; Yoshida and Nagato, 2011; Sugiyama *et al*., 2019; Refahi *et al*., 2021), thanks to massive research on model organisms such as *Arabidopsis thaliana*, *Antirrhinum majus* and rice (*Oryza sativa*). Adam *et al*. (2005) explored the floral development in oil palm using light and scanning electron microscopy. Our knowledge base is further supplemented by studies in closely related palm species American oil palm, *Elaeis oleifera* (de Farias *et al*., 2018) and Coconut palm, *Cocos nucifera* (Perera *et al*., 2010).

An interesting line of enquiry in the field of oil palm reproductive development is the potential use of oil palm inflorescences as tissue culture explants. Each oil palm inflorescence has an infinite number of generative flower meristems. This meristematic tissue may be used for meristem cultures, as has been done in coconut palm (Weckx *et al*., 2019). Similar efforts are underway in oil palm, to develop novel more efficient propagation techniques (Zulkarnain *et al*., 2019; Panggabean *et al*., 2021).

2.3.2.1. Flowering Behaviour and Sex Determination

Floral initiation occurs after a series of defined triggers following the plants' decision to flower, which is influenced by multiple factors such as species, ecotype, age of plant, day length, and other environmental conditions. Oil palms enter the reproductive phase of development soon after seedling establishment, as indicated by the emergence of first bunches two to three years after field planting. They then continue to produce inflorescences in an acropetal sequence in the axils of their subtending leaves continually and indeterminately through its vegetative extension, a behaviour common in palm family termed pleonanthy. The influence of photoperiod however is observed as seasonal peaks in production (Legros *et al*., 2009a; Adam *et al*, 2011; Combres *et al*, 2013).

Individual inflorescences of oil palm take over two years from floral initiation to maturity. Two third of this period is consumed for expansion of the inflorescence meristem to form the inflorescence structure whilst only the last one third contributes to development of the individual flowers (Adam *et al*, 2005).

Sex determination, decision on the sex of the flower or inflorescence (Figure 2.6), occurs early on in inflorescence development. Even though many of the key signals are structurally conserved, sex determination follows diverse pathways in flowering plants. Genetic predispositions as well as, environmental, metabolic, and hormonal factors such as drought, carbon reserves, and gibberellins, respectively are thought to be involved in sex determination in oil palm (Legros *et al*., 2009b; Adam *et al*., 2011; Jaligot, 2018). The mechanisms behind sex determination and genetic and

environmental factors affecting sex ratio (number of female flowers borne by a palm) have been investigated by many as it is an important yield parameter (Oettli *et al*., 2018; Rhebergen *et al*., 2019).

2.3.2.2. Determination of Floral Organ Identity

The scheme for the future floral organ development is laid down by the patterning process within the flower meristem (Lohmann and Weigel, 2002, Plackett *et al*., 2018). Different plant species are unique in their inflorescence architecture, flower size, shape, colour, number of floral organs, symmetry, presence of nectar, time of anthesis, receptiveness of reproductive organs and so forth. These in turn decide their type of breeding system, pollination vectors and seed dissemination mechanisms. Despite this mesmerising diversity, the underlying structure of the flowers is remarkably conserved. The floral primordium of a perfect or hermaphrodite flower develops into four organ types: sepals, petals, stamens and carpel, arranged in concentric whorls namely calyx, corolla, androecium and gynoecium (Theißen, 2001; Kramer, 2006; Causier *et al*., 2010).

Floral organ identity determination in higher plants can be explained by the ABCDE model (Figure 2.11). According to this model the identity of organs that develop in each whorl is defined by the activities of five different clades of genes coding for transcription factors in the MADS-box family namely *SQUAMOSA* (class A), *DEFICIENS* (class B), *GLOBOSA* (class B), *AGAMOUS* (classes C and D), and *AGL2-like* (class E) (Krizek and Fletcher, 2005).

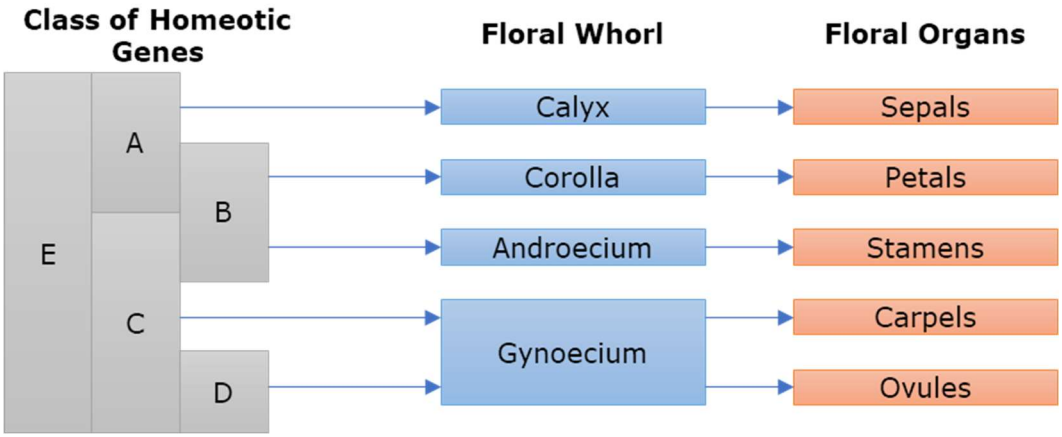

Figure 2.11 ABCDE Model of Floral Identity Determination. Identity of floral organs in the four concentric whorls of a hermaphrodite flower is determined by the different classes of homeotic genes as depicted in the diagram. Class A activity confers sepal identity in whorl 1, Calyx. Class A and class B activity confers petal identity in whorl 2, Corolla. Class B and class C activity confers stamen identity in whorl 3, Androecium. Class C activity confers carpel identity in whorl 4, Gynoecium. Class C and class D activity confers ovule identity within Gynoecium. Class E activity is required for the specification of each organ type. Adapted from Krizek and Fletcher (2005).

The outermost whorl of sepals is specified by class A gene activity, the second whorl petals by a combination of A and B, the third whorl stamens by a combination of B and C, the fourth whorl carpels by C, and ovule identity by C and D. The floral context is affirmed by class E activity and thus is required for all the whorls (Figure 2.11; Krizek and Fletcher, 2005). Disruption of one or more of these genes results in homeotic floral mutants, where the right organ is produced in the wrong position.

The putative MADS-box genes involved in floral organ identity of oil palm has been functionally characterised by RNA in-situ hybridisation and ectopic expression in transgenic *Arabidopsis* plants (Adam *et al*., 2007). Their spatial and temporal patterns of expression during the development of inflorescences suggest the mechanism involved in oil palm resembles the generic ABCDE model (Adam *et al*., 2007).

Fifteen different oil palm MADS-box genes have been identified and named according to their sequence affinities including *EgSQUA1* (A class and/or

meristem identity); *EgGLO2* and *EgDEF1* (B class); *EgAG2* (C or D class); and *EgAGL2-1* (E class). No A function *SQUA* genes have been found in oil palm consistent to other monocots. *EgSQUA1* may be involved in meristem identity but not A function (Adam *et al*., 2006; 2007; Jaligot *et al*., 2011). In 2018 a genetic pathway of reproductive development in oil palm was published by Rival (2018) accounting for the roles of the various floral genes.

2.3.3. Study of Oil Palm Reproductive Development

Due to the obvious economic implications of successful development of oil palm flowers and fruits various researchers have looked at the structural and molecular aspects reproductive development in oil palm. Previously, the morphological changes during normal inflorescence development in seed derived oil palm were studied using microscopic techniques (van Heel *et al*., 1987; Adam *et al*., 2005). Adam *et al*., (2005) also made limited comparisons to abnormal flower development in mantled. Since then, most research focussed on molecular analysis of the genetic and epigenetic mechanisms involved in the normal and abnormal reproductive developmental process (See section 2.4; Jaligot *et al*., 2011; Shearman *et al*., 2013a, 2013b; Rival, 2018; Sarpan *et al*., 2020; Ooi *et al*., 2020). Over the years researchers have looked at whole genome expression patterns as well as organ specific expression of genes. In 2020, Ooi *et al*., conducted transcriptomics of micro-dissected staminodes and early developing carpels from female inflorescences (Ooi, *et al*., 2020).

Biotechnological and genomic molecular analysis of reproductive developmental stages also required accurate identification of developmental

events and methodical approaches for sampling. Firstly, owing to the time consuming and labour-intensive nature of oil palm inflorescence sampling and histological techniques, efficiency and accuracy of practices are important. Secondly, to ensure efficient comparison accurate identification and classification of stages (Chapter 6) is also required. However previous literature is lacking in standardised protocols for selection (Chapter 3) and preparation of samples (Chapter 5) for comparative studies within and between clones.

In previous literature frond number has been used as a field reference for inflorescence developmental stages (Adam *et al*., 2007). Sarpan *et al* (2015) proposed a model for developmental stage prediction based on age of palms and lengths of inflorescences. Both methods are only partially effective in classification of developmental stages for accurate comparison of developmental events within and between clones. In this thesis the morphological features of oil palm inflorescences, and their component structures (spikelets, floral triads and individual flowers) are characterised in detail by visual characterisation and histological study of developmental stages. Data thus generated is used for standardisation of sampling and histology protocols (Chapter 5) and for the preparation of a comprehensive developmental classification (Chapter 6). Said classification is employed for the preparation of a reproductive developmental series that enables comparison between phenotypes within and between clones (Chapter 7).

In this study detailed characterisation of inflorescence developmental stages were done up to anthesis, and the developmental classification allows identification of key developmental events using visual cues. Trends

in the industry suggest future breeding may also use tools of industry 4.0 for the identification of developmental stages. DB *et al*., (2020) proposed use of machine learning approaches for the classification of oil palm female inflorescences anthesis stages for the prediction of pollination period. Methods that use thermal parameters were evaluated for their applicability in precision agriculture (DB *et al*., 2020).

2.3.4. Development of Fruit Bunch

Mature female inflorescences (roughly between leaf stages F17 and F20) after successful pollination and resultant fertilisation develop into fruit bunches over the next 5-6 months. Individual fruits increase in size and weight, and the embryo matures within the kernel during this period. The oil content of the kernel and mesocarp continues to increase till the ripe fruits detach from the bunch. Ripe fruit bunches are seen ready for harvest at around F30 (Figure 2.12; Adam *et al*., 2005).

The ripe fruit bunches are ovoid (Figure 2.12). Bunches from the ten-year-old palms reach more than 50cm in length and 35cm in breadth. The younger the palm the smaller and lighter the fruit bunches. A typical fruit bunch consists of a thick peduncle on which spikelets are spirally arranged. Spikelets bear individual fruits armed with spines, which are formed by the fibrous bracts (Figure 2.12 B; Corley and Tinker 2008).

Fruits are spherical to ovoid or elongate sessile drupes (Figure 2.12 C). The pericarp of the fruit consists of the outer exocarp (a reddish-orange skin), mesocarp (or pulp rich in oil) and endocarp (hard stony shell surrounding the kernel). In most cases, the seed may have only one kernel as a result of the abortion of the other two ovules in the tri-carpellate ovary. Abnormal seeds may rarely occur, giving rise to even five kernels.

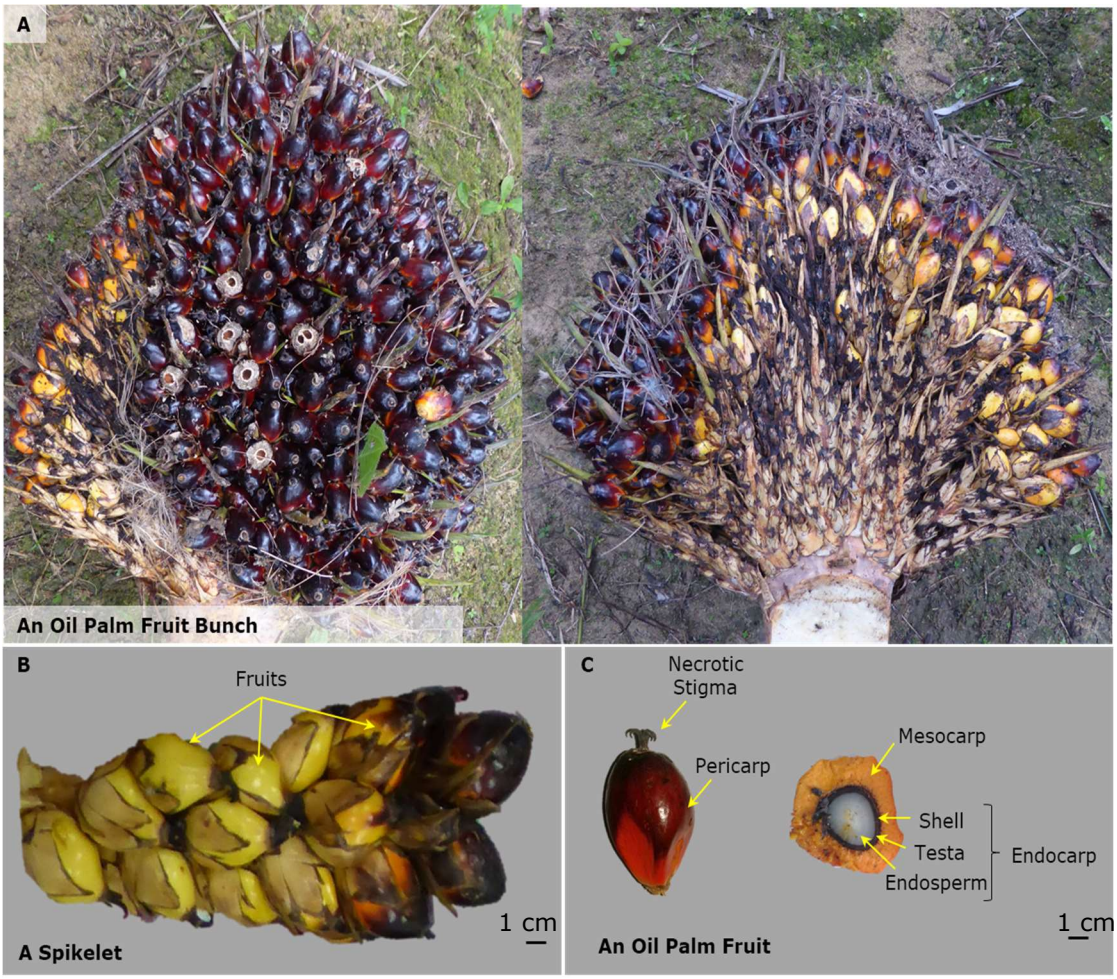

Figure 2.12 Oil Palm Fruits. (A) Anterior (Front) and Posterior (Back) views of an oil palm fruit bunch. Less pigmentation and smaller unfertilized fruits are seen on the posterior sidewhich is pressed against the leaf base. (B) An oil palm spikelet bearing ripening fruits. (C) An oil palm fruit and it's cross section showing different parts of the fruit. The stigma become necrotic, and the pericarp turns reddish orange in colour as the fruit develop. The orangish mesocarp and white endosperm are rich in oil content and are the sources of palm oil and palm kernel oil respectively.

The kernel consists of the shell (when present), the testa (or skin), a solid endosperm (that is rich in oil) and the developing embryo (Figure 2.12 C). The fruit development takes place from about the 15th to 19th day after anthesis and oil formation takes place towards the end of maturity during which the shell hardens, and the embryo becomes viable (Corley and Tinker 2008).

E. guineensis has three primary fruit forms: Thick-shelled '*Dura*', shell-less female-sterile '*Pisifera*' and the hybrid of the two, thin-shelled '*Tenera*'. The *Tenera* palms have better oil yields than *Dura* and are used for commercial palm oil production in all of South-East Asia (Corley and Tinker 2008; Jaligot, 2018). *Tenera* fruits have thin shell (0.5-4.0mm) with medium to high mesocarp content (60-95%). There is a prominent fibre ring in its mesocarp. A naturally occurring abnormal fruit type had been infrequently reported called *Poissoini* or *diwakkawakka* characterised by the fleshy outgrowths surrounding the main fruit. These outgrowths called 'supplementary carpels' develop from the rudimentary lobes of androecium and often containing shell and kernel themselves (Hartley, 1988; Jaligot, 2018).

2.3.4.1. Significance of Bunch Parameters

The economic produce of oil palm is the oil from mature fruits. So, the bunch yield is regularly recorded at the plantations, usually in terms of the number of bunches harvested and their weight. The number of bunches produced per palm depends on leaf production rate, sex ratio and abortion/bunch failure rate. The size and weight of the bunch depend on numerous factors including genotype, age of palm and environmental factors. The weight of bunches is in turn influenced by the inflorescence architecture, that is the

number of spikelets, number of flowers per spikelet, and also fruit set and the weight of fruit and stalk. These are studied in detail in relation to their yield contribution. Also, methods are available for sampling fruits from bunches for assessment of oil content and yield parents (Rance *et al*., 2001; Harun and Noor, 2002).

Mother palms are selected based on high Fresh Fruit Bunch yield, bunch parameters like fruit to bunch ratio, oil to bunch ratio, shell to fruit ratio, and other morphological and dry matter parameters. For selecting pollen parent *Pisifera*, selfed / interse population of high yielding *Tenera* (T x T) is grown in the field and evaluated for 10-12 years (See section 2.2). Palms showing female sterility and other bunch parameters with good general combining ability are selected as *Pisifera* parents (Rance *et al*., 2001; Harun and Noor, 2002).

Developing and developed bunches provide a picture of successful or unsuccessful reproductive development. In chapter 4, bunch characteristics of normal and mantled ramets are analysed by visual scoring. Data thus generated is used for the accurate characterisation of the abnormal phenotype, which has not been done previously.

Interestingly in oil palm interspecific hybrids (*Elaeis oleifera* Cortes x *Elaeis guineensis* Jacq.) parthenocarpic fruits may be preferred owing to the high labour cost involved with assisted pollination required for the production of fertile bunches. To that end, Daza *et al*., (2020) evaluated potential methods of inducing parthenocarpy in hybrid palms by the application of plant hormones. Mantled abnormality is associated with fruit sterility and production of parthenocarpic fruits. Perhaps unravelling the mechanism

behind parthenocarpy associated with mantled may help uncover potential approaches for inducing parthenocarpy as a beneficial trait. The relationship between fertility of fruits and the severity of mantled was also investigated using visual scoring data (Chapter 4).

2.3.5. Oil Palm Pollen

Oil palm is an open-pollinated crop. During male inflorescence anthesis, more than 100,000 flowers open gradually in acropetal fashion releasing pollen. Pollen production continues for four to five days. Pollination occurs via wind and insects. Fennel like fragrance emitted from the inflorescences, both male and female, attracts the insect pollinators. Starch in the pollen acts as a food source for weevils that inhabit the male inflorescence during the pollen shedding period (Tandon *et al*., 2001).

Oil palm pollen is *trichotomosulcate*, that is it has three distal sulci (furrows or slits) (Figure 2.13). It is triangular with rounded angles and is distal opening. The size of oil palm pollen ranges from 31 µm to about 32 µm (Zeven, 1964; Harley and Baker, 2001; Ibrahim *et al*., 2012).

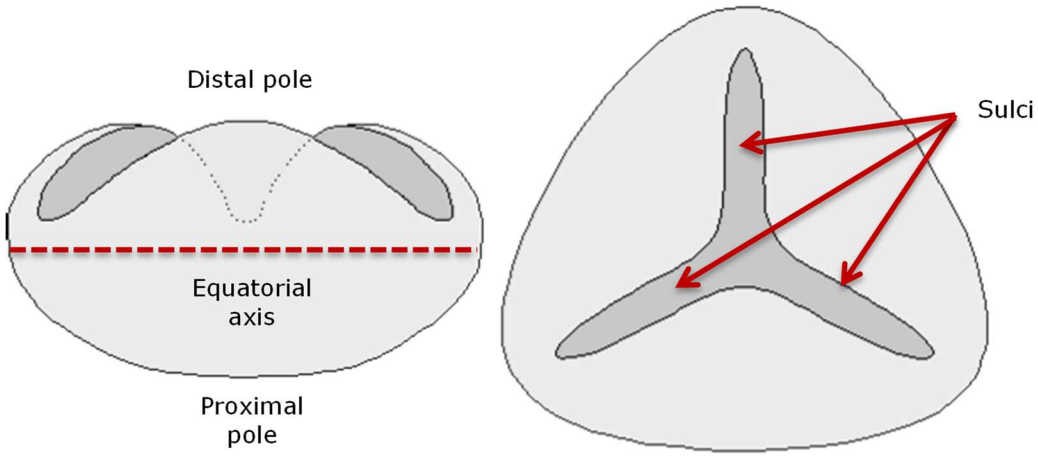

Figure 2.13 Illustration of *Trichotomosulcate* Pollen of Oil Palm. From the left, side and top view of an oil palm pollen is depicted showing the three distal sulci. Adapted from Erdtman (1943).

In plantations, pollen is routinely collected from superior male palms and is stored for assisted pollination. Like other allogamous species, the production of improved oil palm seedlings and ortets involves controlled pollination between parents with superior complementary traits. Commercial *Tenera* seed production hinges on such controlled pollination. The male inflorescence is bagged in a superior *Pisifera* parent and cut down with the bag intact. The pollen is collected and desiccated on silica and stored in common freezers (about -4°C) for a duration up to 6 months (Communications with plant breeding station, Paloh).

Pollination could prove ineffective due to the short lifespan of pollen, loss of quality of pollen, and poor synchronisation between pollen shed and stigma receptivity. To avoid these shortcomings, a comprehensive understanding of the pollination process of oil palm and proper handling of pollen from the field to the laboratory, where it is conserved for subsequent use, are necessary. Further pollen grains have an important role in modern breeding techniques and germplasm conservation. There are only a few previous studies on functional quality of oil palm pollen (Tandon, 2007; Myint *et al*., 2012) and none in relation to abnormal floral phenotypes. This gap in research is addressed in chapter 8.

2.4. MANTLED SOMACLONAL VARIANT

According to Hartley (1988), mantled is a naturally occurring floral abnormality. It is described by the feminization of androecium of flowers of both sexes, to form a ring of supplementary carpels (Figure 2.14). The supplementary carpels, characteristic of the phenotype, are also known as supernumerary carpels or pseudocarpels, denoting they are in excess of the

normal number of carpels or are not functional carpels respectively. The sterility of fruits depends on the severity of mantled phenotype. In less severely mantled palms, the carpel is usually fertile, but in severely mantled palms, they are parthenocarpic and sterile (Ooi *et al*., 2019). Mantled is a rare occurrence in seed-derived palms, however, its occurrence was aggravated in clonal propagation, and this has restricted the use of clonal material in commercial planting. Continuous improvements undergone by the oil palm tissue culture technique have been successful in decreasing the occurrence of this floral abnormality to manageable levels of < 5% in the clonal material. Now, combined with stricter culling techniques during tissue culture, nursery production, and field planting, it has been possible to reduce the percentage of abnormalities seen in the field. However, we still lack a complete understanding of the origin and expression of this phenotype and its correlation to tissue culture (Jaligot *et al*., 2011; Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020).

The ubiquitous manifestation of the mantled variant in clonally propagated oil palm was first brought to the attention of a conference organized by the International Society for Oil Palm Breeders (ISOPB) in 1985 and was later reported by Corley *et al*. (1986). This floral developmental abnormality of oil palm clones also results in poor pollination, partial or complete sterility, parthenocarpic fruit set, defective fruit ripening, reduction of overall fruit size, low oil yields and severe bunch failure. The symptoms, however, transpire at varying degrees of severity in flowers of the same inflorescence, in inflorescences of the same palm, and in ramets of the same clone and different clones (Jaligot *et al*., 2011).

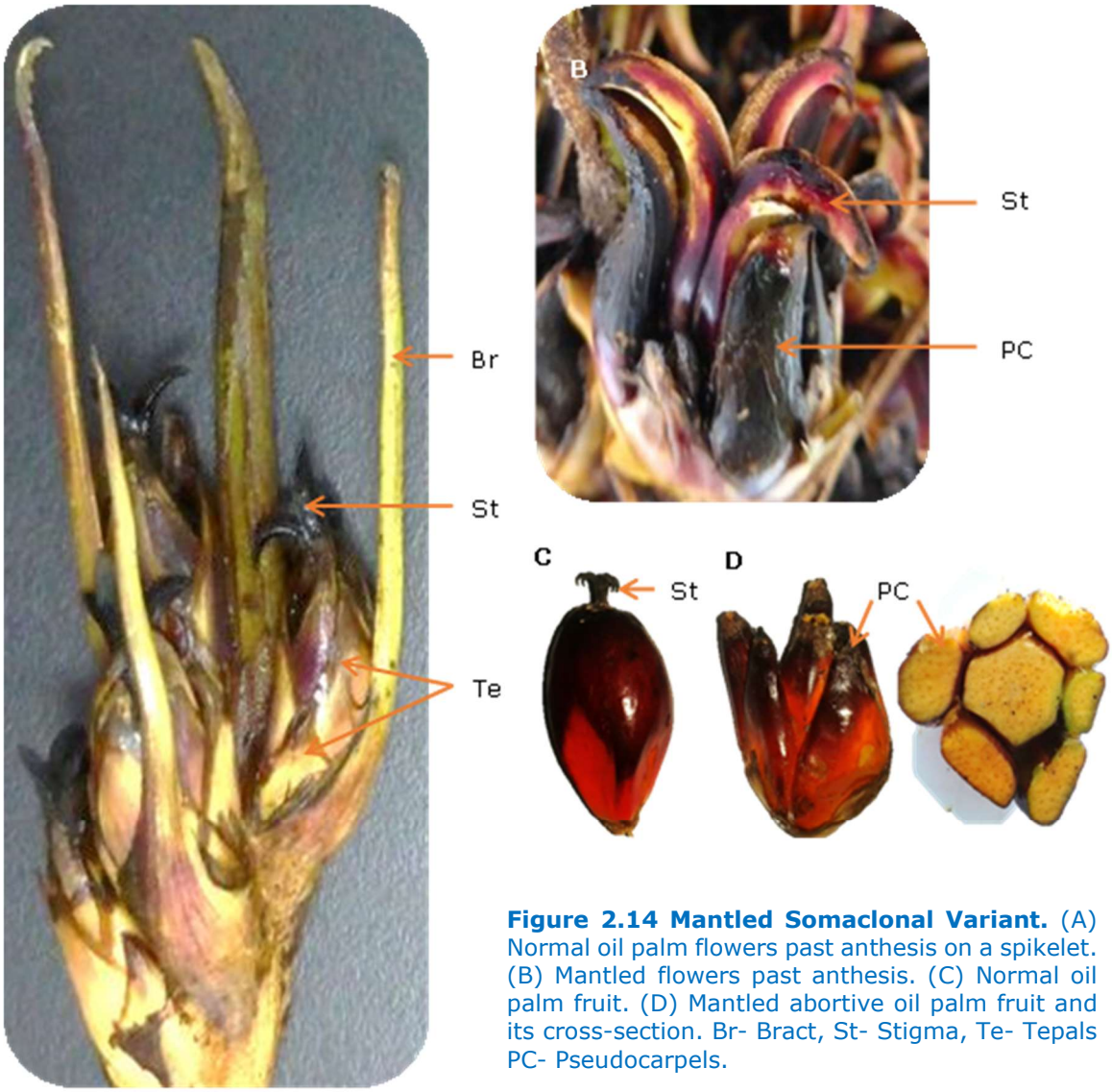

Figure 2.14 Mantled Somaclonal Variant. (A) Normal oil palm flowers past anthesis on a spikelet. (B) Mantled flowers past anthesis. (C) Normal oil palm fruit. (D) Mantled abortive oil palm fruit and its cross-section. Br- Bract, St- Stigma, Te- Tepals PC- Pseudocarpels.

2.4.1. Genetics and Epigenetics of Somaclonal Variations

A somaclonal variation is a phenotypic or genotypic modification that arises from *in vitro* culture. It could arise from changes in the genome or epigenome. Changes in the genome may be a mutation of the genetic material, that is DNA in the nucleus or organelles of plant cells. Genetic change may be in chromosome number (aneuploidy, polyploidy or mixoploidy) or chromosome structure (deletion, inversion, duplication, translocation, activation of transposable elements or point mutation). Observable traits or phenotypes associated with genetic mutations are

expected to follow Mendelian transmission, an inheritance pattern that follows the laws of segregation and independent assortment, during conventional crossing (Harel *et al*., 2015).

However, the mantled abnormality has been found not to follow Mendelian transmission during conventional genetic crossing (Morcillo *et al*., 2006). Furthermore, according to Jaligot *et al*. (2011), referring to the cytometry and extensive RAPD and AFLP analyses carried out on tissue culture derived oil palm material by Rival *et al*. in 1997 and 1998, there is no evidence of any 'gross genetic defect' that could be linked to mantled abnormality. Interestingly enough, the naturally occurring similar phenotype called *diwakkawakka* was noted to be inherited monofactorially and to be dominant by Adam *et al*. (2005). That is, this trait is governed by one gene with two alleles (monofactorial) with one allele masking or overriding the effect of others (dominance). Non-Mendelian inheritance observed in the case of mantled phenotype indicates there may be a more complex genetic origin involved.

On the other hand, there are a number of aspects of this variation suggestive of an epigenetic origin. Epigenetic change in a chromosome does not alter the DNA sequence but can cause a stably heritable phenotype, by changing how the DNA sequence is read. As a result, they are not distinguishable by conventional structural molecular markers. In the case of mantled variation, Soh *et al*. (2011) noted genetic/clonal differences in susceptibility/tolerance to the mantled abnormality. Further, the occurrence of the abnormality is highly variable among palms of the same clonal origin,

and mantled palms sometimes show spontaneous reversion to the normal phenotype both of which are characteristic of epigenetic changes.

Epigenetic changes typically happen post-transcriptionally to the DNA and nuclear proteins and are often reversible. One example of an epigenetic change or epigenetic mark is DNA methylation. During DNA methylation a methyl group is added to the nitrogenous base Cytosine (C) of the DNA molecule. In plants DNA methylation is found in multiple sequences: CG, CHG, and CHH (where C stands for the Cytosine, G for Guanine and H for Adenine, Cytosine, or Thymine). This prevents certain genes from being expressed. Another example is histone modifications. Histones are proteins that the DNA double helix wraps around to form chromosomes. Modifications to the histones such as methylation, phosphorylation, acetylation, and ubiquitylation change the physical structure of the DNA and alter its accessibility to proteins that "read" genes.

Epigenetic changes may also occur due to the activation of quiescent Transposable Elements (TEs) and retrotransposons. TEs, also known as "jumping genes" are DNA sequences that, when active, can move (or jump) from one location in the genome to another. Retrotransposons copy and paste themselves into different locations in the genome using an RNA transposition intermediate (Kaeppler *et al*., 2000; Miguel and Marum, 2011; Harel *et al*., 2015).

It is now known that they are an evolutionarily conserved key mechanism by which plant species exhibit a high degree of developmental plasticity. They help plants cope with the changing environment (Chinnusamy and Zhu, 2009; Boyko and Kovalchuk, 2011). Furthermore, modification of plant

genome by time-related changes in different epigenetic marks have been reported at many instances to regulate plant growth and reproduction (Ronemus *et al*., 1996; Kawashima and Berger, 2014; Shafiq *et al*., 2014; Shi *et al*., 2015). For example, vernalization, the induction of flowering as a consequence of exposure of the developing plant or germinating seed to a period of low temperature, is an epigenetically controlled developmental process operative in many plant species. Dennis and Peacock (2007) observed that "the period of exposure to low temperature results, at a time later in plant development, in the transformation of the vegetative meristem of the plant to a reproductive meristem." In *Arabidopsis,* this process is governed by specific modifications of the histones associated with the chromatin of the gene *FLOWERING LOCUS C* (Dennis and Peacock, 2007).

Epigenetic modifications result in reversible changes in the expression of genes which theoretically is reproducible by providing the same conditions. In the case of the above example of vernalization, the epigenetic marks or "the mitotic memory of the vernalized state" is reset in the next sexual generation. But this can be induced again through low temperature exposure.

Epigenetic changes could either be temporary with plants reverting back to normal phenotype relatively easily, or these changes could have inheritable long-lasting effects that last few generations (Feng and Jacobsen, 2011). Meiotic inheritance of the epigenetic mark DNA methylation has been demonstrated in plants. Methylation patterns are maintained through cell divisions as DNA methyltransferases add methylation to newly synthesized

strands using the old, methylated strand as a guide (Iwasaki and Paszkowski, 2014).

Somaclonal variations pose a challenge to maintaining the required genetic fidelity and uniformity in clonally propagated plants. It thus is a stumbling block for both *in vitro* cloning of elite genotypes, selected for their superior characteristics, as well as germplasm preservation of genetic resources. But in some cases, somaclonal variations have helped uncover natural variability that could be utilised for breeding (Miguel and Marum, 2011; Krishna *et al*., 2016). However, the incidence of the deleterious mantled somaclonal variant in tissue culture progeny has held back the commercial expansion of high yielding clonal palms for decades (Soh *et al*., 2011; Jaligot *et al*., 2014; Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020).

2.4.2. Origin of Mantled Phenotype

Since the early 1990s, researchers have been studying the mantled variant (Figure 2.14). Parallel to continued improvement in tissue culture techniques researchers invested in finding a diagnostic tool for early detection of the mantled variation. Studies also focussed on explaining its origin by targeting either molecular changes or looking at the suspected genes/pathways (Jaligot *et al*., 2000, 2004, 2014; Matthes *et al*., 2001; Eeuwens *et al*., 2002; Tregear *et al*., 2002; Kubis *et al*., 2003; Alwee *et al*., 2006; Cullis *et al*., 2007; Rival *et al*., 2008, 2013; Beule *et al*., 2010; Ooi *et al*., 2013; Shearman *et al*., 2013a, 2013b; Mgbeze and Iserhienrhien, 2014; Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020).

In search of a means for early detection of mantled, researchers adopted global gene expression analysis via DNA microarray, genetic mapping and candidate gene approach (Jaligot *et al*., 2011). The publication of the genome sequence (Singh *et al*., 2013) accelerated the identification of genomic regions, especially differentially expressed regions in mantled variants, through comparative genomics analyses. Subsequently, Ong-Abdullah *et al*. (2015) performed a genome-wide search for alterations in DNA methylation that were tightly linked to the mantled trait. This led to the detection of methylation changes associated with mantled in a previously overlooked gene fragment. This differentially methylated fragment is a transposable element now called ’Mantled’ that has homology to rice *Karma LINE* elements. *MANTLED* lies in the intron of a B class homeotic gene of oil palm (*EgDEF1*) that specifies pistil/stamen identity.

MANTLED transposon encodes a 'splice acceptor' site, a sequence that directs splicing in the RNA transcript. When the methylation is reduced, possibly due to tissue culture in this case, the *Karma* splice site becomes active. Although the mechanisms underlying this specificity are not known, the alternatively spliced *EgDEF1* transcript *(kDEF1*) accumulates during flower development and encodes a truncated *EgDEF1* protein (Ong-Abdullah *et al*., 2015). Ong-Abdullah *et al*. (2015) reported increased expression of the truncated transcript of *EgDEF1*, *kDEF1* in female inflorescences at organogenesis; and proposed a causal effect in the development of pseudocarpels, in the place of staminodes in mantled phenotype.

KARMA test which detects differentially methylated sites associated with mantled abnormality is the current means for early detection of the

deleterious phenotype (Ong-Abdullah *et al*., 2018). Palms included in this study were tested using the initially published method of methylation detection on the *Karma* transposon using the restriction enzyme *RsaI* (referred to as the *KARMA* assay).

2.4.3. Mantled a B Class Homeotic Mutant?

Mantled abnormality exhibits alteration of the organ identity. Previous reports show that in mantled flowers fertile (in the case of male) and sterile (in the case of female) androecium are converted into carpelloid structures (Figure 2.14). Similar phenotypes have been reported in the B class homeotic mutants of model monocot and dicot species. Floral homeotic mutants, where expression of one or more of the floral homeotic genes are disrupted, results in wrong floral organs developing in the wrong location/whorl. Two examples from model plant *Arabidopsis thaliana* are presented in figure 2.15.

Alwee *et al*. (2006) noted the resemblance between mantled phenotype (Figure 2.16) and "the transition of stamens into supernumerary carpels in *Antirrhinum majus* as the result of a B-type mutation (described by Zachgo *et al*., 1995)". There are three putative B function genes identified in Oil palm (Adam *et al*., 2007). Of these *EgGLO1* and *EgGLO2* belonging to the *GLO* subfamily, and *EgDEF1* belonging to the *DEF* subfamily, are expressed in whorl two and three, that is inner perianth/petals and androecium consistent with B class function (Figure 2.15; Adam *et al*., 2006; 2007; Jaligot *et al*., 2011).

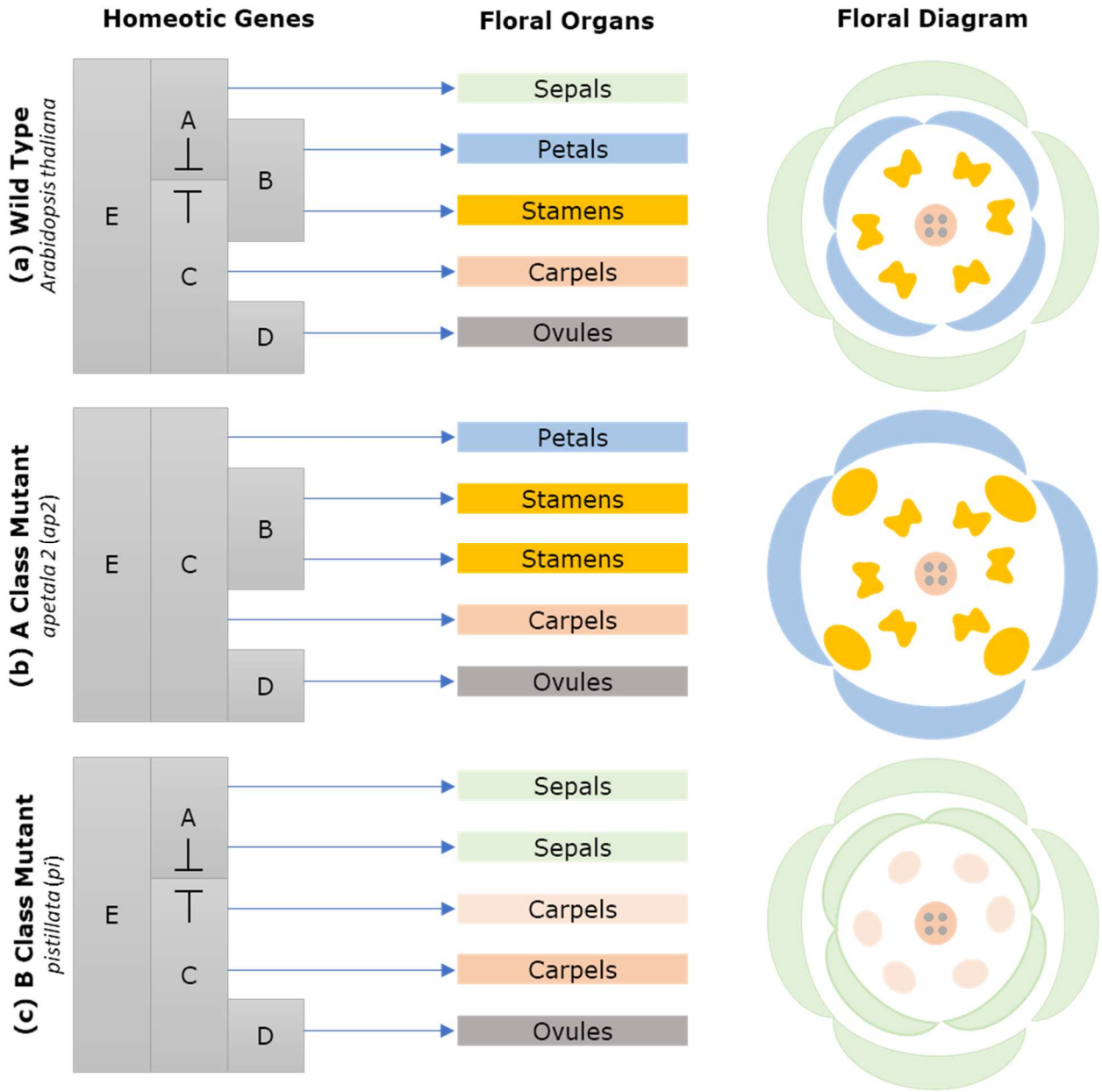

Figure 2.15 A and B Class Homeotic Mutants of *Arabidopsis thaliana*. Each row shows from the left, activity of homeotic genes, resultant floral organs in each whorl and floral diagram depicting floral morphology. (a) The wild type *Arabidopsis thaliana* flower consists of four concentric whorls of floral organs namely Sepals, Petals, Stamens and Carpels. Ovules develop within the carpels. The floral organ identity in each whorl is determined by the activity of ABCD and E classes of homeotic genes. Class A activity confers sepal identity in whorl 1, class A and class B activity confers petal identity in whorl 2, class B and class C activity confers stamen identity in whorl 3, class C activity confers carpel identity in whorl 4, and class C and class D activity confers ovule identity. Class E activity is required for the specification of each organ type. Class A and class C genes inhibit each other. (b) In the homeotic mutant *apetala 2 (ap2)* that lacks class A activity, class C activity expands to outer whorls. Flowers consist of carpels in whorl 1, stamens in the whorls 2 and 3, and carpels in whorl 4. (c) In the homeotic mutant *pistillata (pi)* that lacks class B activity, flowers consist of sepals in the whorls 1 and 2, and carpels in the whorls 3 and 4. Adapted from Krizek and Fletcher (2005).

Changes in the expression profile of oil palm putative B class gene were confirmed by Beulé *et al*. (2010) while looking at differentially expressed genes between normal and mantled inflorescences using microarray hybridisation. The decrease in the mRNA levels of both the B class genes, *EgDEF1* and *EgGLO2* genes, was demonstrated in male and female inflorescences displaying the mantled phenotype compared with those from true-to-type palms. However, low mRNA levels could not be detected consistently throughout the development of the inflorescences.

Petal to sepal transformation in mantled flowers is difficult to detect through visual observation due to the morphological similarity of these floral organs (Figure 2.16). However, spatial expression patterns suggest that the petals and sepals can be distinguished by MADS-box gene expression patterns. *EgGLO2* (B class) is expressed in floral organs of whorl one- sepals. Whereas *EgDEF1* (B class) and *EgAGL2-1* (putative E class) gene expressions are detected in the floral organs of whorl 2- petals but not in the sepals. Thus, there are distinct molecular identities for the morphologically similar floral organs of whorls 1 and 2. MADS-box gene expression changes in whorl 2 mantled flowers are similar to *ap3, pi* mutants of *Arabidopsis* and indicates transformation of petals to sepals (Alwee *et al*., 2006; Adam *et al*., 2007).

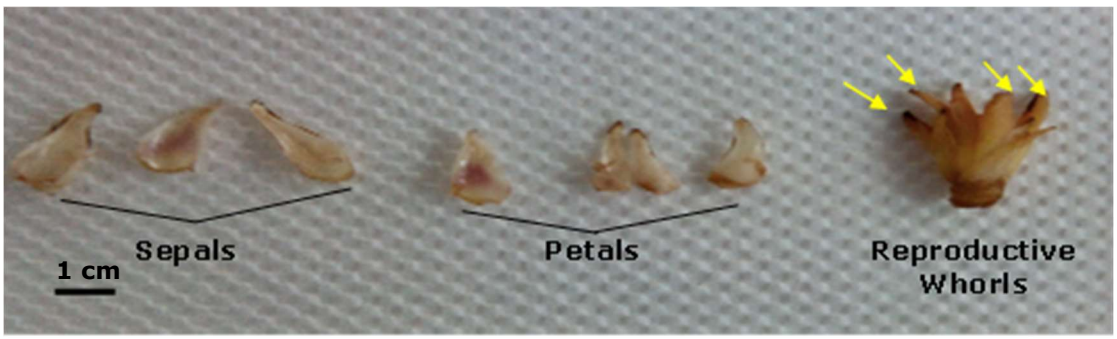

Figure 2.16 Floral Organs of a Mantled Pistillate Flower. A young, mantled flower (before anthesis) with tepals removed showing carpelloid structures known as pseudocarpels (yellow arrows) similar to B class homeotic mutants.

During the evolution of bisexuality, the homeotic conversion of stamens to carpels (microsporophyll to megasporophyll within a pollen cone) is thought to have occurred due to the differences in maximal expression levels of B and C class floral organ identity genes and the competition of their gene products for partners in multi-numeric complexes (Baum and Hileman, 2006). Thus, changes in transcriptional activity of homeotic genes specifying the stamen and carpel structures (B class) may be causal or instrumental in the formation of the mantled phenotype.

Involvement of DNA methylation in the regulation of the B class ortholog in oil palm *EgDEF1*, as an implication of reactivation of neighbouring transposable elements (the *Koala* retrotransposon present in the 5th intron of *EgDEF1* and *copia* element located upstream from the promoter) in the hypomethylated state, was explored by Jaligot *et al*. (2014). It was shown improbable since the gene is essentially unmethylated and the methylation pattern of it or the retrotransposons are unchanged in the mantled variant. Only in 2015, the third transposon *karma* was discovered, and its relevance in the formation of the shorter alternative *EgDEF1* transcript was noted (Jaligot *et al*., 2014, Ong-Abdullah *et al*. 2015).

Other genes in the reproductive pathway have also been studied in relation to mantled abnormality (Shearman *et al*., 2013a, 2013b). One of the most significant of them was *LEAFY*, an upstream regulator in the reproductive development pathway which is expressed at higher levels in pseudocarpel initials (or primordia). In *Arabidopsis*, the interaction of the *LFY-UFO* complex with *AP3* promoter activates the expression of *AP3* for petal and stamen specification. *AP3* and *PI* then autoregulate their expression

(Kaufmann *et al*., 2009, 2010; Winter *et al*., 2015; Silva *et al*., 2016; Plackett *et al*., 2018; Ooi *et al*., 2019). The transcript isoform of oil palm B class gene *EgDEF*, *kDEF1* detected at organogenesis in mantled inflorescences probably leads to a truncated protein (Ong-Abdullah *et al*. 2015), this may break the positive feedback loop, therefore resulting in low levels of *EgDEF1/EgGLO1* expression and thus necessitating elevated *LFY* expression (Vetaryan *et al*., 2018). A summative study on the role of MADS-box genes and the possible genetic mechanism involved in the differential expression of floral developmental genes in mantled phenotype is Rival (2018).

Furthermore, previous reports show that genes involved in primary hormone responses, DNA replication and repair, chromatin remodelling, RNA mediated DNA methylation stress response, redox regulation and many others are differentially expressed in the mantled mutant (Beulé *et al*., 2008; Rival *et al*., 2009; Ooi *et al*., 2019).

Previous studies largely discount the variability of the mantled phenotype, even though an increasing amount of results point to differential expression patterns associated with it. For instance, the total protein profiles of fruit and floret samples of 100% abortive mantled and 50% fertile mantled palms, showed notable differences from the normal (Yaacob *et al*. 2013). Yaacob *et al*. (2013) noted not only the difference in band intensities but also bands unique to either mantled or normal phenotypes indicating that in mantled variants some proteins produced during the normal developmental process were no longer produced, while some additional unique proteins were produced in the abnormal phenotype. So, in the

current study efforts were made to characterise the mantled phenotype in detail to remove ambiguities in comparison (Chapter 4). Further, in this study comparisons are made throughout the developmental process alongside an equivalent sample from a normal ramet belonging to the same clone (Chapter 7).

2.4.4. Insights from Related Species

Mantled phenotype resembles B class mutants *pistillata (pi)* of model plant *Arabidopsis thaliana*. The similarity between the homeotic phenotypes suggests highly conserved mechanisms controlling floral organ identity. The phenotype of *pi* is caused by a recessive mutation in the floral homeotic gene *PISTILLATA (PI).* However, the source of mantled phenotype is more complex.

Jaligot *et al* (2011) suggested the epigenetic mechanisms involved in the origin of mantled may be similar to that involved in the formation of '*Hose in Hose'* floral phenotype of primrose (*Primula vulgaris*). *Hose in Hose* is long-documented phenotypic instability, that shows dominant homeotic conversion of sepals to petals. The phenotype has been shown to be associated with the up-regulation of both the B-class homeotic genes of Primula, *PvDef* and *PvGlo,* in the first floral whorl. More significantly a retrotransposon insertion in the *PvGlo* promoter has been found to be associated with the epigenetic changes and up-regulated expression of *PvGlo*.

An interesting comparison to consider is with mutants that occur in a closely related *Arecaceae* palm species *Phoenix dactylifera L.* (date palm). A

somaclonal variant similar to the mantled phenotype of oil palm is found to occur in date palm. Referred to as "abnormal" or "low fruit setting phenotype", it is found widely among tissue culture derived date palms of the cultivar '*Barhee*'. While the common phenotypic expression involves the formation of parthenocarpic fruitlets with three carpels and reduced fruit set, in severe cases multicarpel flowers and fruitlets have been reported. The abnormal flowers (and fruitlets) of the phenotype have six or eight carpels instead of the normal three as seen in mantled phenotype of oil palm (Attaha and al-Saadi, 2015; Abd-Elhaleem *et al*., 2020).

Similar to pistillate flowers of oil palm, pistillate flowers of the date palm is also composed of three carpels and upon effective pollination a single carpel develops into a fruit and the other two degenerate. Parthenocarpic fruitlets with three carpels are observed to develop when pollination is not efficient. This is interesting since there is no previous research looking at the effect of mantled phenotype on pollen quantity, quality or the process of pollination and fertilization. Some reports suggest homeotic transformation of staminate flowers, in the male inflorescences of mantled oil palm ramets, in which case mantled palms produce no pollen at all (Adam *et al*., 2007). However, there is little information available on mantled male inflorescences or mantled pollen. These are examined in chapters 9 and 8 respectively.

Abnormal *Barhee* date palms showed a frequency of occurrence in tissue culture (approximately 5%, but more common in some sources) and reversion patterns (in severe cares 50% reverting to normal in 10years from planting) very similar to mantled oil palms. Attaha and al-Saadi (2015)

conducted anatomical and hormonal studies of fruit set in normal and abnormal date palms. Their results revealed structural deformities including the separation of the style from the stigma and ovary, the disintegration of the ovules, and the absence of zygotes in the abnormal phenotype. More recently Ali-Dinar *et al*. (2021) investigated the effect of pollination interventions on normal and abnormal date palms and observed strong correlations between levels of indigenous plant hormones and the abnormal phenotype. This observation also parallels previous reports on mantled oil palm (Jaligot *et al*., 2011).

The precise origin of the abnormal date palm phenotype is still unknown. But Cohen *et al* (2004) observed that "the similarities between 'Mantled' oil palm and *Barhee* off-types, and the lack of detected genetic variation in both cases, suggest that an epigenetic mechanism expressed by an altered DNA methylation pattern may be responsible for the formation of the *Barhee* off-type phenotype (Cohen *et al*., 2004; Abd-Elhaleem *et al*., 2020; Ali-Dinar *et al*., 2021).

2.5. SUMMARY REMARKS

Oil palm is an exceptionally productive oil crop, with a key role to play in the global supply of vegetable oils. Innovative and efficient breeding methods are required for yield maximization in oil palm, to meet the increased global demand for vegetable oil while limiting the environmental impacts (Soh *et al*., 2003; Qaim *et al*., 2020). Large-scale oil palm propagation is difficult due to the unique botany (single shoot apical meristem, no natural vegetative propagation methods). Micropropagation via tissue culture allows the multiplication of superior progeny *in vitro* and

the storage of germplasm elites. The mantled somaclonal variant has restricted the usage of clonal progeny in commercial cultivation for decades (Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020).

Mantled palms develop abnormal fruits with reduced oil content. They are identified and removed in the commercial plantations at fruiting age, with substantive loss in investments. From the practical necessity of understanding and preventing the emergence of the pervasive variation the mantled phenotype has been studied extensively. Significant progress has been made towards elucidating the molecular mechanisms behind mantled. The publication of the oil palm genome sequence accelerated the research efforts (Jaligot *et al*., 2011; Singh *et al*., 2013; Ong-Abdullah *et al*., 2015).

Mantled mutants also present an opportunity to study the reproductive pathways of oil palm. Its phenotype resembles B class floral homeotic mutants of model species in its phenotypic expression and has been linked to the hypomethylation of a transposable element within the oil palm B class gene Eg*DEF1* (Ong-Abdullah *et al*., 2015). However, the specific origin of the variant phenotype and the epigenetic misregulations involved still eludes us. What is known of mantled variant today is not a straightforward cause-effect relationship. All the data generated through research from the last 30 years have made a Gordian knot of interacting pathways of homeotic genes, epigenetic marks, plant growth regulators, and so on. Even with the high throughput tools now available, finding the root cause(s) where they all converge and deciphering the underlying mechanism from there remains a challenge.

While researchers (Adam *et al*., 2005) have examined the mantled phenotype through microscopic techniques, none have done this across the whole reproductive developmental process alongside comparable normal stages. Existing models for prediction (Adam *et al*., 2005; Sarpan *et al*., 2015) of inflorescence developmental stages are limited in terms of the categories generated and the developmental events captured. Further, there are gaps in the morphological characterisation of the Mantled phenotype as seen in inflorescences and fruit bunches. This is highly significant due to the complexly variable expression pattern of the phenotype. Also, previous literature is lacking standardised methodologies for sampling and processing of oil palm inflorescences and characterisation of mantled fruit bunches. Here, considerations are taken for the selection of comparable ramets (Chapters 3, 4) and equivalent developmental stages (Chapter 6) to enable comparisons of normal and mantled inflorescence samples across the developmental stages (Chapter 7). The methods proposed for sample selection and processing reduces ambiguities, improve efficiency, and also enable comparisons between ramets of the same clone and different clones (Chapter 5).

In previous literature, there were no standardised protocols available for histological examination of oil palm pollen. Moreover, oil palm pollen from mantled sources has not been studied before. In fact, there is very little information available on the effect of mantled phenotype on male inflorescences. The present research is a step towards addressing these gaps (Chapters 8 and 9), in the hopes of furthering our understanding of oil palm reproductive development and the mantled somaclonal variant.

CHAPTER 3
DETERMINATION OF GENOTYPIC AND PHENOTYPIC IDENTITY OF OIL PALM RAMETS

3.1. INTRODUCTION

Objectives:

- Identification of comparable normal and mantled ramets belonging to different clones and molecular and morphological characterisation of ramets for accurate determination of genotypic and phenotypic identity.

Systematic analysis of reproductive development in oil palm requires accurate comparisons between samples of known genotypic and phenotypic identity. Hence careful considerations were taken in selection and characterisation of plant material for the study.

3.1.1. Considerations in the Selection of Sampling Range

Primarily, it was proposed that to understand the differences in reproductive development in normal and mantled phenotypes, comparable samples were required from inflorescence developmental stages across the full range of reproductive development. Consequently, two aspects of oil palm were considered while selecting the plant material for the research.

1. **Stability of phenotypes (normal and mantled) of interest**: Even though oil palm enters the reproductive phase soon after seedling establishment and is estimated to start bearing by 2 years in the field, the mantled phenotype is unpredictable and variable in younger palms (Adam *et al*., 2005; Jaligot *et al*., 2011). In addition, various physiological abnormalities are observed in young palms, due to

environmental stresses such as close planting, or water stress. Hence older palms, 10 years of age in the field, were found better suited for comparisons between the two phenotypes.

2. **Accessibility to a good range of floral developmental stages:** As the oil palm inflorescences develop over a period of two to three years enclosed with the prophyll and peduncular bract at the base of developing leaves, only a limited number of stages could be extracted without damaging the palm permanently (Adam *et al*., 2005). That is, to study younger developmental stages destructive sampling methods are required. However, older mantled ramets available in the field were part of multiple studies and trials and could not be used for destructive sampling. Hence young ramets from clonal screening nurseries were also included in the study, to investigate younger developmental stages.

Therefore, two types of sampling methods were used: non-destructive sampling of mature palms of stable phenotype and destructive sampling of young palms for extraction of younger developmental stages. Comparisons were done between mantled and normal palms of same age to eliminate age related distortions.

3.1.2. Considerations for Genotypic Conformity

Secondly, clone to clone differences, if any, had to be discounted and the differences observed had to be corroborated across clones. So, comparisons were proposed within and between clones. Each mantled palm was compared to a normal counterpart from the same clone. The results were then verified with mantled-normal pairs from other clones. To do this the clonal identity or origin of the ramets were confirmed using SSR fingerprinting (genotyping using SSR markers).

Simple Sequence Repeats (SSRs) or microsatellites are highly polymorphic repetitive sequences ubiquitously present in the eukaryotic genomes. SSRs are abundant in oil palm genome with high variation in repeat numbers between different clones (Singh *et al*., 2007).

SSR fingerprinting is widely used in genetic diversity studies and for marker assisted genome wide selection, thanks to its ease of use, low developmental costs and transferability between labs. Primer pairs are designed to flank and amplify sections of repetitive DNA sequences in the genomic DNA. Examination of the size of these amplified alleles can be used to distinguish between genotypes within narrow and mixed populations such as commercially cultivated oil palm clones, crosses, and sibs (Chee *et al*., 2015).

In addition, to expand the scope of the study plant material was selected from a wide genetic background. The palms selected for the study belonged to clonal lineages derived from some of the major breeding populations, as follows:

1. ***Deli*** is the thick-shelled *Dura* variety derived from the original four Bogor palms in Java. *Deli Duras* provide the mother palms for almost all major oil palm commercial hybrid seed production programs. ***Dumpy Dura*** is a short variant of the *Deli*.

2. ***AVROS*** *Pisifera* palms with high fresh fruit bunch production and high oil extraction rate usually serve as the pollen donor. *AVROS* population was derived from the *Tenera* palm SP540, progeny of Djongo (best) palm at Eala Botanical Garden in Zaire, which was crossed with *Teneras* at Bangun Bandar experimental station and subsequently back crossed with SP540 selfs. They exhibit vigorous growth, precocious bearing, thin shell, thick mesocarp and high oil yield attributes.

3. ***Yangambi*** population was derived from breeding of open-pollinated seeds from the Djongo palm and from *Teneras* in Yawenda, N'gazi and Isangi. They show high vigour, have short palm feature, big fruits and high oil yield. *AVROS* and *Yangambi Tenera x Pisifera* (TxP) crossing produces ***Yangambi-AVROS*** which is a superior pollen donor for seed production. ***Dumpy-Yangambi-AVROS*** *Pisifera* palms are also used for seed production and are derived from breeding *Dumpy-AVROS* of TxP and Yangambi-AVROS of TxP.

3.1.3. Phenotypic Considerations for Reasonable Comparisons

Thirdly, to ensure standardised comparisons, the mantled status of the selected palms was determined. Previous publications had documented mantled phenotype to be highly capricious and varied in its expression (Jaligot *et al*., 2011). Hence, it was necessary to determine the degree of mantled phenotype in the palms to ensure reasonable comparisons. The mantled status of the selected palms was evaluated by the *KARMA* Assay (Ong-Abdullah *et al*., 2015), as well as phenotyping in the field by meticulous scoring. The phenotyping regime was designed for small fruit bunches of young palms and big fruit bunches of old palms for effectual characterisation of the phenotypic expression of the palms.

Lastly, possible environmental effects on the phenotype/inflorescence development were reduced by ensuring comparisons between palms of the same replicates.

As a result of these considerations, comparisons were possible between normal and mantled palms belonging to the same clone, at each stage of inflorescence development. The results were then verified among different clones. Comparisons were also conducted between different degrees of mantled phenotype.

3.2. MATERIALS & METHODS

3.2.1. Plant Material

Plant samples were collected from two estates at Paloh and Kekayaan, in Johore, South of Peninsular Malaysia. All the palms selected for the study were clonal palms (known as ramets) derived from *Tenera* hybrids by somatic embryogenesis. These clonal materials were from field trials conducted by Advanced Agriecological Research Sdn. Bhd. (AAR) Oil Palm Breeding Research Station.

From the total available plant resources for the study, palms were selected based on the multiple criteria and methods as summarised in Figure 3.1. Twenty-four mature palms (10 years of age) belonging to 3 clones and ten young palms (3 years of age) belonging 2 clones were carefully chosen after 3 rounds of meticulous selection.

Even though the mother palms (known as ortets) could not be accessed, information of their genetic background was retrieved (Table 3.1). These parental lines make up the major genetic backgrounds of the oil palm populations in Malaysia.

Table 3.1 Genetic Backgrounds of the Different Clonal Lines Included in the Study. Clone lineage is represented by letters A or R indicating primary clone or reclone respectively followed by a serial number. Genetic background is shown in terms of the parental lines of the ortet palm. In a cross the left is the maternal parent and right the paternal parent (pollen donor).

Clone lineage	**Genetic Background**
Lineage R291	*Deli x Yangambi AVROS*
Lineage R295	*Deli x Yangambi AVROS*
Lineage A229	*Deli x Cameroon*
Lineage A366	*Deli x Yangambi AVROS*
Lineage A478	*Deli x Dy AVROS*
Lineage A202	*Dumpy Deli x Cameroon*
Lineage A204	*Deli x AVROS*
Lineage A218	*Deli x unknown*

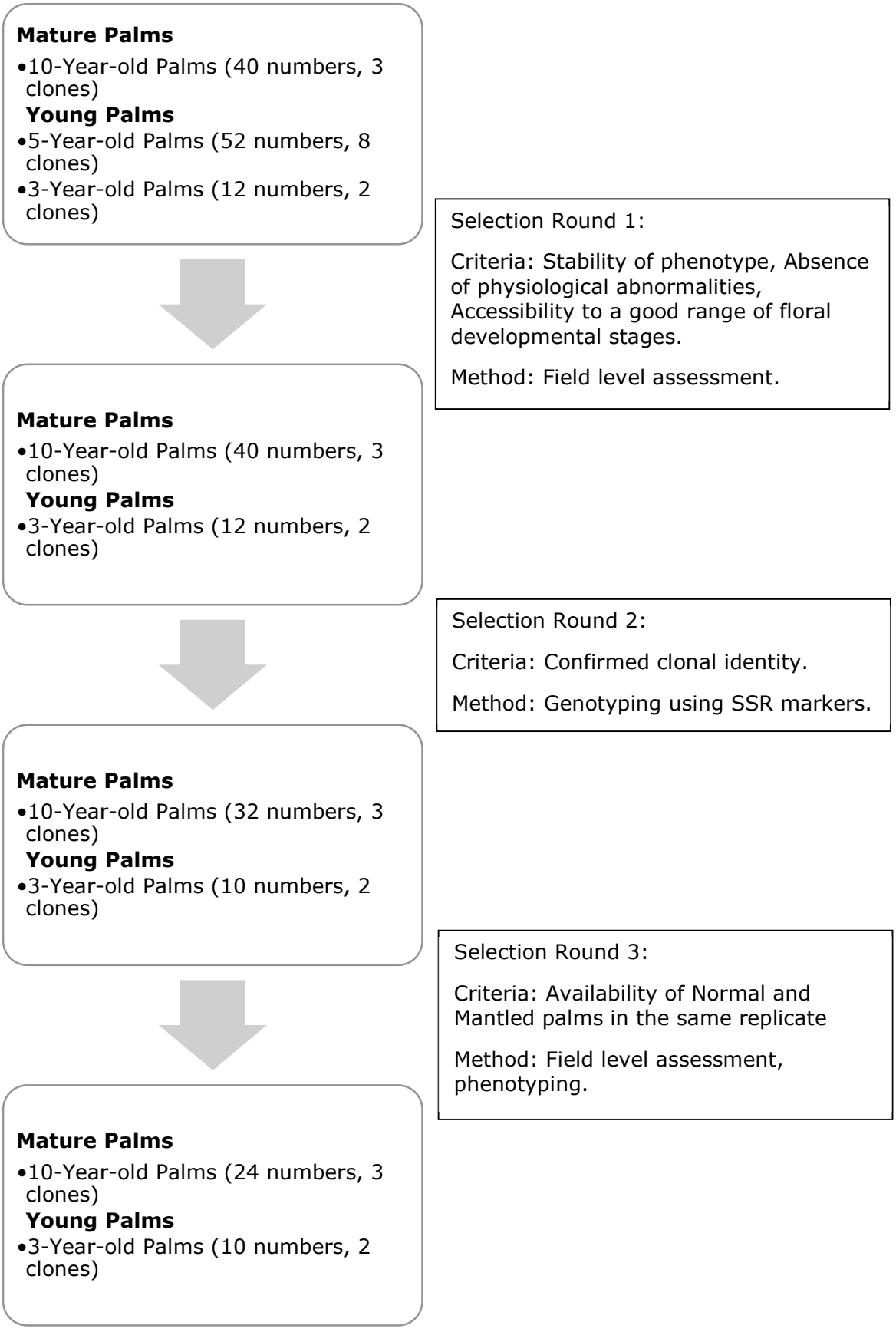

Figure 3.1 Scheme of Selection Process of Plant Material. Number of clones and palms belonging to each age group is shown on the left and the selection criteria and method employed at each stage is shown on the right.

3.2.1.1. Samples types

Different types of samples were collected from the different groups of palms for genotyping (Chapter 3), phenotyping (Chapter 3,4) and subsequent study of reproductive developmental stages (Chapter 5,6,7) as shown in table 3.2.

Table 3.2 Details of Sample Types and their Use. Types of Samples collected from palms belonging to each age category, and the purpose for sampling is detailed.

Sample Group / Sample type	Mature Palms (Ten-Year-Old)	Young Palms (Three-Year-Old)
Leaf sample	Used for DNA extraction, SSR fingerprinting, *KARMA* Assay.	Used for DNA extraction, SSR fingerprinting, *KARMA* Assay.
Un-ripe bunch	Used for Phenotyping.	Used for Phenotyping.
Inflorescence sample	Non-destructive sampling.	Destructive Sampling.

3.2.2. Naming Convention

In oil palm young fronds open at the centre of the crown and progress spirally around the central spear (Figure 3.2). Young leaves open at the

Figure 3.2 Arrangement of Fronds Around the Central Spear and the Numbering of Fronds Adapted. Orange crescents represent leaf bases arranged spirally around the crown on the trunk.

centre of the crown next to the spear (seen as a green spine). They then grow in spirals of eight. Leaf 1 is the youngest fully open leaf, on the top of the palm. The leaf beneath Leaf 1 is Leaf 9, the leaf below Leaf 9 is Leaf 17 and so on. About two new leaves appear per month. New leaves appear faster in young palms.

Inflorescences develop at the base of these fronds. The developing inflorescence stages and fruit bunches were thus numbered according to the position of the subtending leaf in the crown.

> That is, frond number depicted as leaf stage **F##** represents the developing inflorescences or bunch where **##** is the chronological advancement of the subtending leaf from the spear.
>
> Inflorescence within the youngest fully open leaf, on the top of the palm (frond 1), was thus designated leaf stage F1 or simply F1. Next older inflorescence (from frond 2) was designated leaf stage F2 or F2 and so on.

3.2.3. Oil Palm Leaf Sampling

Leaf samples were used for DNA extraction (Section 3.2.4) for the purpose of genotyping (Section 3.7) and *KARMA* assay (Section 3.8). The following protocol was followed for uniformity in sampling.

The palms and their canopy were photographed for documentation before collection of samples. The youngest fully opened frond next to the spear, frond one, was cut down with a harvesting tool (Figure 3.3 A). The spines towards the base of the frond indicate whether it is fully opened or not. Four leaflets from either side were collected from the middle portion where the

ridge of the main stem becomes an inverted V shape, indicating the vicinity to the vascular bundles (Figure 3.3 B).

Figure 3.3 Oil Palm Leaf Sampling Procedure. Frond One is cut down with a harvesting scythe (A), the middle 8 leaflets are taken (B), trimmed from either side (C) and packed in plastic bags with labels(D)

Diseased or insect eaten leaflets were avoided, and dirty ones were wiped with a towel. The basal and apical portions of the leaflets were trimmed (Figure 3.3 C) and resultant rectangular samples of approximately 25-30cm length were packed in plastic bags with labels and fastened with rubber bands (Figure 3.3 D). Each label carried details of the field, palm number,

frond number, date of collection and phenotype, the same details were written on the plastic covers once more with a permanent marker. Leaf samples were kept in air-conditioned environment except during transit.

In the lab, the midribs of the leaflets were removed and discarded. Any leaves damaged by pathogens or insects (Leaves with orange spotting or eaten or infected tissues) were also discarded. Selected leaves were then washed under tap-water to remove debris. Surface sterilization was achieved by soaking leaflets in 3% (v/v) Clorox solution for three minutes and thereafter washing in two changes of filter water and one change of type 3 water (RO Water). They were air-dried and packed into aluminium foil parcels in zipper bags with label cards. Samples were subsequently stored at -20ºC. Care was taken to keep the leaves as intact as possible to reduce their degradation.

3.2.4. DNA Extraction

Genomic DNA was extracted from leaves using CTAB method where high DNA yields (750-1000ng/µl) were required for DNA banking or ambient temperature transport of DNA samples. Genomic DNA Mini Kit was used for DNA extraction where lower DNA yields (around 100ng/µl only) were sufficient. Extraction using Genomic DNA Mini Kit was quicker and yielded sufficient quantity and quality of DNA for SSR fingerprinting.

3.2.4.1. DNA extraction using CTAB Method

Genomic DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987) with minor modifications. Modified CTAB lysis buffer was prepared immediately before starting the extraction process, by dissolving 2% (w/v) polyvinylpyrolidone-

40 (PVP-40) in 2x CTAB buffer [2% (w/v) CTAB, 100 mM Tris-HCl pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 140 mM sodium chloride (NaCl)] by continuous stirring at 65 °C followed by addition of 1% (v/v) Ascorbic Acid, 1% (v/v) Diethyldithiocarbamic acid, sodium salt trihydrate (DIECA) and 0.4%(v/v) 2-mercaptoethanol. Protective equipment (Mask and goggles) were worn while handling PVP-40 and addition of components to the solution was carried out under the fume hood.

Leaf samples were retrieved from -20 freezer and placed on dry ice. Leaflets were reclaimed from the packaging and the deteriorated tissue of extremities were discarded. The leaflets were cut into small pieces and were ground to fine powder in liquid nitrogen using sterile (autoclaved) pre-chilled pestle and mortar. Five grams of ground samples were scooped, using sterile pre-chilled spatulas, into 50mL centrifuge tubes pre-labelled with proper identification of the DNA source (clone/treatment number-palm number). Centrifuge tubes were kept on dry-ice till all the samples were ground.

Twenty millilitres of modified CTAB buffer was added to the centrifuge tubes and the sample powder was fully dispersed in the buffer by vortexing. The centrifuge tubes were then incubated in preheated water bath at 65°C for 1 hour. The tubes were inverted gently periodically to mix. After incubation tubes were allowed to cool for 5-10 minutes and an equivalent amount of 24:1 (v/v) chloroform: isoamyl alcohol (C:I) was added. The tubes were shaken in the orbital shaker (N-Biotek, Inc) for 15 minutes and then allowed to stand for 10 minutes for the leaf samples to settle at the interface between the upper aqueous phase and lower chloroform phase. Ten millilitres of the upper aqueous phase was retrieved from them and added

to the new centrifuge tubes containing 10 ml C:I. The new tubes were also shaken for 15 minutes, followed by centrifugation (Sigma 3-18K) at 10,000 x g for 15 minutes at room temperature (25 ºC). Eight millilitres from the upper aqueous phase was transferred into a third set of labelled 50 ml centrifuge tubes. One and a half times by volume of cold absolute ethanol was added to them. They were mixed by gentle inverting. Strings of DNA were visible at this stage. The centrifuge tubes were kept at -20°C overnight for precipitation.

On the following day, the DNA pellets were gently scooped out and transferred into the new 15 ml centrifuge tubes with 10ml wash buffer [70% (v/v) absolute ethanol and 10 mM ammonium acetate]. They were shaken at room temperature for 1 hour and afterwards centrifuged at 10,000 x g for 3 minutes at 4°C in precooled centrifuge. Supernatant was gently decanted, and pellet was drained on tissue paper. The samples were allowed to air-dry on the bench. One millilitre TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) was added and the pellet was dissolved by gentle tapping. The tubes were kept at 4°C overnight or until DNA was fully dissolved. Successful extraction of gDNA was confirmed by gel electrophoresis (section 3.2.5).

RNase Treatment: To remove the RNA contaminants from the genomic DNA samples 1.25 µl of RNase (10 ng/ml) was added to the samples on the next day of extraction. The samples were then incubated at 25ºC overnight. Next, an equal volume of ethanol was added to the samples and they were kept at -20ºC overnight. On the following morning DNA was re-precipitated by centrifugation in precooled centrifuge. The pellet was dried for 30

minutes in the desiccator. It was then re-suspended in TE buffer and gel electrophoresis confirmed quality (section 3.2.5).

3.2.4.2. DNA Extraction Using Genomic DNA Mini Kit

Genomic DNA Mini Kit (Plant) from Yeastern Labs (Cat. Nos. YGP 10-S/YGP10) was used for fast genomic DNA extraction from oil palm leaves.

Fifty to hundred milligrams of frozen plant tissue samples were ground in liquid nitrogen to a fine powder with sterile pre-chilled pestles and mortars. The homogenized powder was transferred into labelled microcentrifuge tubes. Four hundred microlitre GP1 Buffer (or GPX1 Buffer) and 5 µl RNase A (10 mg/ ml) was added to each sample and mixed by vortexing. Tubes were incubated at 60ºC for 10 minutes, with inverting every 5 minutes.

After incubation, 100 µl GP2 Buffer was added. Following vortexing, to mix the contents thoroughly, the tubes were incubated on ice for 3 minutes. Filter Columns were placed in 2 ml Collection Tubes and the mixture from previous step was applied to it. After centrifugation (1 minute at 1,000 x g) the filter columns were discarded and clarified supernatant in the collection tubes carefully transferred to new microcentrifuge tubes. One and a half volumes of GP3 Buffer (isopropanol added) was added to the cleared lysate and mixed immediately by vortexing for 5 seconds. GD Columns were placed in 2 ml Collection Tubes and the mixture (including any precipitate) from the previous step was added to the GD Columns, 700 µl at a time. The tubes were centrifuged at full speed (16,000 x g) for 2 minutes, then the flow-through in 2 ml Collection Tube was discarded.

Four hundred microlitres of W1 Buffer (ethanol added) was added to the GD columns and they were centrifuged at 14,000 x g for 30 seconds. Next,

600µl of Wash Buffer (ethanol added) was added followed by centrifugation at 14,000 x g for 30 seconds. The flow-through was discarded, the GD Columns were placed back in the 2 ml Collection Tubes and the column matrix was dried by spinning at full speed (16,000 x g) for 3 minutes.

Dried GD Columns were transferred into clean, labelled 1.5 ml microcentrifuge tubes and DNA was eluted out using 50 µl of elution Buffer preheated at 60ºC. Elution Buffer was added to the centre of the column matrix, allowed to stand for 3-5 minutes until it was absorbed by the matrix and then centrifuged at 16,000 x g for 30 seconds to elute purified DNA.

3.2.5. Quality Check by Agarose Gel Electrophoresis

The quality of genomic DNA obtained via the different extraction methods was checked by gel electrophoresis. In the case of CTAB method, quality check was done before and after RNase treatment.

The samples were prepared by adding 20% (v/v) loading dye to the DNA extracts. They were then run on a 0.8% (w/v) agarose gel (Vivantis) containing 5% (v/v) SYBR® Safe DNA gel stain (Invitrogen) in 1x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.0) at 90 V for 45 minutes. Visualization was done on a FluorChem HD2 Multi Image II (Alpha Innotech).

3.2.6. Quantity Determination Using Nanodrop

The quantity of DNA was estimated using Nanodrop (Nanodrop 1000 Spectrophotometer, ThermoScientific). Samples were incubated at 37°C and homogenised by pipetting up and down. Three microlitre of each sample was loaded on the nanodrop. Spectrophotometer absorbance readings were performed at 230nm, 260nm, 280nm and 320nm. Each sample was measured twice.

The 260/230 and 260/280 ratio were calculated to check for protein or solvent contamination. A 260/280 ratio greater than 1.8 and 260/230 of 1.5 to 1.8 was ensured. A lower 260/280 indicates protein contamination and a lower 260/230 indicates salt or solvent contamination in the DNA samples.

Concentration of DNA (ng/µl) in the samples were estimated using the following formula

Concentration (µg/ml) = A_{260} *reading* × *dilution factor* × *50µg/ml*

Where A_{260} is the spectrophotometer absorbance reading at 260nm and dilution factor is the factor by which the original solution is diluted.

3.2.7. Genotyping Using SSR Markers

Genotyping/ fingerprinting of the plant material to verify their clonal identity was done using M13-tailed primers of SSR markers (Chee *et al*., 2015). The forward SSR primer were synthesised with a M13 (-21) tail, 5'-TGT AAA ACG ACG GCC AGT-3' (18bp), at the 5'end. During the PCR amplification, the universal IRDye®-labelled M13 (-21) primer was incorporated into the PCR reaction. Thus, the PCR products were labelled with the fusion primer and standard reverse primer.

A total of 24 SSRs were first screened using a representative subset of samples (two DNA samples each from the 3 different clones included in the study), five control genotypes and negative control (Table 3.3). The objective of this screening process was to eliminate markers that are monomorphic for the studied clones. Results of primer screening is included in appendix 1. Results revealed two monomorphic markers, and one SSR that showed no amplification.

Table 3.3 Sample Subset and Controls Used in Primer Screening and Genotyping. DNA samples used for primer screening included a subset of the 3 mature clones included in the study (1-6), five control genotypes representing diverse oil palm populations (7-11) and a negative control which offered no DNA for amplification (12). The controls (7-12) were also used in genotyping of selected palms to confirm their genetic identity.

No.	Sample ID
Subset of Samples	
1	R291/ 23
2	R291/ 16
3	A299/ 69
4	A299/ 68
5	R295/ 27
6	R295/ 24

No.	Sample ID
Controls	
7	C986/40
8	0702004
9	0707020
10	0611015
11	126/37
12	negative control

Following the initial screening, twenty SSRs with high levels of polymorphism for the clones studied were selected. The selected SSRs were used to develop unique fingerprints for 52 palms belonging to the 5 different clones: namely R291 (mature), R295 (mature), A229 (mature), A366 (young) and A478 (young). The forward and reverse SSR primer sequences used for the PCR amplification are listed in (Table 3.4).

All the DNA samples were diluted to give a final concentration of 10ng/ µl for the PCR. The composition of PCR reaction mixture (10 µl) is detailed in Table 3.5. The 5 control genotypes and negative control were included with each set (one set of controls per primer pair) to give a reasonable degree of confidence in the genetic identity of the sample set.

The PCR amplification was performed in Veriti 96-well Thermal Cycler (Applied Biosystems, USA). The programmes used varied slightly with the two different DNA polymerases used and are included in table 3.6 and table 3.7. The final IRDye®-labelled products were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were cast with 0.25mm spacers without any bubbles (for recipes see appendix 5). Sizing standard was included on either side of the gel asymmetrically. The gels were visualised with an infrared dye detection system NEN 4300 DNA Analyser (Li-COR Biosciences, USA).

Table 3.4 Sequence Details of SSR Primers used for Genotyping. Twenty SSR primer pairs (Tm=52°C) as listed below were used to generate unique fingerprints of selected palms to confirm their genetic identity. Primer ID is in keeping with MPOB and Billotte *et al*. (2005) for primers 1 to 3 and 4 to 20 respectively. Primer sequences are written from 5′ to 3′ direction. Location of the SSR where known is included as the chromosome number according to sequenced genome EG5 (Oil Palm SSR Resource Interface-OPSRI, MPOB).

No	Primer ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size of Amplicon (bp)	Location
1	sMg00025	GAGGAGGAGGGGAGAAGAGT	AAATACCATTCAGAGAAAGCAC	198	EG_Chr16
2	sMg00042	CCGAATAGAAGAGGAAAGAATA	AGGTTTGGTGGAGAAGTGTT	247	EG_Chr5
3	sMg00108	ACGAAACAGAGGCATAGAGACT	ACAATTAAACAGCAACGCTAGA	186	EG_Chr7
4	mEgCIR00369	GGGTAGCAAACCTTGTATTA	ACTTCCATTGTCTCATTATTCT	206	EG_Chr14
5	mEgCIR03428	GACAGCTCGTGATGTAGA	GTTCTTGGCCGCTATAT	175	EG_Chr3
6	mEgCIR03649	TTTAGAGGACAAGGAGATAAG	CGACCGTGTCAAGAGTG	284	-
7	mEgCIR03544	AGCAGGGCAAGAGCAATACT	TTCAGCAGCAGGAAACATC	188	-
8	mEgCIR02595	TCAAAGAGCCGCACAACAAG	ACTTTGCTGCTTGGTGACTTA	184	EG_Chr2
9	mEgCIR03358	CCAAGGAACAACATAGA	GTTCCCATCCTATTAGAC	208	-
10	mEgCIR00783	GAATGTGGCTGTAAATGCTGAGTG	AAGCCGCATGGACAACTCTAGTAA	296	-
11	mEgCIR03389	GTCCATGTGCATAAGAGAG	CTCTTGGCATTTCAGATAC	93	EG_Chr9
12	mEgCIR03808	CCGCTAACTTGGTATAC	ATTTCCAGCAGCTAATC	190	-
13	mEgCIR02332	GAAGAAGAGCAAAAGAGAAG	GCTAGGTGAAAAATAAAGTT	204	-
14	mEgCIR02492	CATCAAGCATGACTGCAAGTAA	TTCCGAATTTGGATGAATCC	248	EG_Chr6
15	mEgCIR03311	AATCCAAGTGGCCTACAG	CATGGCTTTGCTCAGTCA	176	EG_Chr5
16	mEgCIR02427	GAAGGGGCATTGGATTT	TACCTATTACAGCGAGAGTG	116	-
17	mEgCIR03546	GCCTATCCCCTGAACTATCT	TGCACATACCAGCAACAGAG	286	EG_Chr11
18	mEgCIR00521	GTGACTTTGGGCTGAAT	ACAGCATCTCCAACTCTATC	137	-
19	mEgCIR00177	TGAATGTGTGTGCAATGTGTAT	ATAGTCAATAATCGTAGGAAAATG	114	-
20	mEgCIR03298	GACTACCGTATTGCGTTCAG	GGTTTTGGTTCGTGGAG	137	EG_Chr15

Table 3.5 Composition of PCR Mix Used for SSR Primer Screening and Genotyping. *DNA polymerases TaKaRa Taq™ (Takara, Japan) and HotStarTaq (QIAGEN) were used.

Ingredient	Quantity (µl)
H_2O	1.80
DNA polymerase (5 units/µl)*	5.00
Forward primer (10 µM)	0.40
Reverse primer (10 µM)	0.40
IRDye®-labelled M13 primer 700/800 (1 µM)	0.40
DNA (10ng/ µl)	2.00

Table 3.6 PCR Profile Used for TaKaRa Taq™. TaKaRa Taq DNA Polymerase (Takara, Japan) is a recombinant-version Taq polymerase derived from the *Thermus aquaticus* YT-1 strain. It was used as per manufacturers specifications.

Temperature	Duration	
94°C	4:00 mins	
94°C	0:30 min	x 30 cycles
52°C (Tm)	1:00 min	
72°C	1:00 min	
72°C	10:00 mins	
10°C	On hold	

Table 3.7 PCR Profile Used for HotStarTaq. HotStarTaq DNA Polymerase (QIAGEN) uses a chemically mediated complete inactivation of the polymerase until the initial heat activation (95°C). It was used as per manufacturers specifications.

Temperature	Duration	
95°C	5:00 mins	
94°C	0:30 min	x 30 cycles
52°C (Tm)	1:30 mins	
72°C	1:00 min	
72°C	10:00 mins	
10°C	On hold	

This method offered the possibility of combining the data sets and hence was chosen over capillary electrophoresis platform. Allele scoring was done visually to approximate sizes. The genotyping summary is included in Table 3.10 and the complete genotyping results are included in Appendix 2.

3.2.7. *KARMA* Methylation Assay

The methylation qPCR detection protocol descripted by Ong-Abdullah *et al*. (2015) was strictly followed for the *KARMA* methylation assay using the published primer sequences (Table 3.8) and the restriction enzyme *RsaI* (New England Biolabs).

Table 3.8 Sequence Details of Primers Used for *KARMA* Assay. Primer IDs and sequence information are from Ong-Abdullah *et al*. (2015). Letters L and R represent left (forward) primer and right (reverse) primer respectively. Primer sequences are written from 5′ to 3′ direction

Primer ID	Sequence Information
KarmaMeth_L1	AGTCAGCACTAGACCATCTTCT
KarmaMeth_L3	TTCGAGGTGGTGTCAATGGA
KarmaMeth_R2	TTGAAAGCAAGCTCTGTGGA
KarmaMeth_R4	TGAATTGAGGGAGAAGGAATGT

As a control the restriction enzyme, and conditions for restriction digestion were first tested on the plasmid PCR8/GW/TOPO RUS4 WD (size= 4377bp). The plasmid was a PCR8/GW/TOPO Topovector from Invitrogen with an insert of *ROOT UV-B SENSITIVE4 (RUS4)* of *Arabidopsis* in the wrong direction (WD). The plasmid had M13 forward and reverse primers and 13 *RsaI* restriction sites (GT^AC sites) which was useful for tests in this study.

Hundred micrograms of Genomic DNA was digested with *RsaI* under standard conditions (table 3.9). An equal amount of genomic DNA was mock treated in a reaction without the enzyme.

Table 3.9 Conditions Used for Restriction Digestion of DNA Samples with *RsaI*. The restriction enzyme *RsaI* (New England Biolabs) was used as per manufacturer's instructions. 1x NEBuffer (New England Biolabs) consisted of 50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, and 100 µg/ml Recombinant Albumin (pH 7.9 @ 25°C). Incubation for restriction digestion was done at 37°C for 16 hours. Heat inactivation was not required for this enzyme.

Ingredient	Quantity
RsaI restriction enzyme	1 µl
DNA	1 µg
10x NEBuffer	5 µl
Total Reaction Volume	**50** µl

The qPCRs were carried out using each of the mock-treated and enzyme-digested samples. The qPCR amplifications were performed in triplicates.

The ΔCt value and DNA methylation density were calculated as follows

ΔCt value *= the digested Ct - the mock Ct*

ΔCt value for each sample were calculated as the average of the triplicates.

Percentage methylation *= 2(−ΔCt(digested − mock))*

3.2.8. Bunch Selection and Phenotyping

An under-ripe bunch, outside the sampling range (>F18), was selected for phenotyping by visual scoring (See chapter 6 for classification and plate 6.1 for images). Drying of stigma and shape of fruit indicative of the presence of an enlarging ovary were considered the mark of the transition from mature inflorescence to fruit bunch.

The bunch was cut down with the harvesting tool. The spikelets and individual fruits were detached using a secateur and were used for phenotyping. Weight, length and width of the bunch and number of spikelets and fruits were recorded when possible. Whole bunch scoring was adapted for young clones. The method was further adapted for mature palms. Since a typical fruit bunch of a mature palm has 1500–2000 fruits, it was not practical to count and score them all. Thus, a representative subset of fruits was selected. Twenty spikelets were selected from different parts of the bunch, that is the top, middle, bottom, left and right from both the front and back sides of the fruit bunch (Figure 3.4).

Establishment of Mantled Phenotype: Any palm with mantled fruits in its bunches were classified mantled. A score of '100% mantled' was established for palms that produced bunches with only mantled fruits, irrespective of fertility.

Figure 3.4 Sampling Technique Used for the Scoring of Large Bunches from Mature Palms. Figure shows the anterior and posterior (pressed against the frond base) side of the bunch and selected spikelets and their positions. Spikelet morphology (length and size of fruits borne) varied slightly across the bunch. A representative subset of fruits from these selected spikelets were used for visual scoring. The bar represents 10cm.

Mantled percentage was calculated as follows for a fruit bunch:

$$\textbf{Mantled Percentage (\%M)} = \frac{number\ of mantled\ fruits\ (M)}{total\ number\ of\ fruits\ scored\ (Ts)}$$

Where, $Ts = M + N = T - U$

Ts the total number of fruits scored (Ts), M the number of mantled fruits or the number of fruits showing homeotic transformation, N the number of normal fruits, T the total number of fruits per bunch and U the unscorable fruits.

Mantled percentage of a palm were calculated by finding the average of all bunches scored. That is, the mantled percentage of a palm is the average of %M calculated for all the bunches of the palm that was scored. Mean values were supplemented with standard deviation where applicable.

$$\textbf{Standard deviation (}\sigma\textbf{)} = \sqrt{\frac{\Sigma(X-\mu)^2}{n}}$$

Where X is the value of observation, μ is mean and n is the number of observations.

3.3. RESULTS AND DISCUSSION

3.3.1. Genotyping and Selection of the Plant Resources

SSRs reveal polymorphisms between ramets belonging to different clones, whereas ramets of the same clone being genetically identical have the same SSR fingerprint.

Out of the 52 palms genotyped, 42 were found to be genuine clones (selection round 2, figure 3.1). All palms belonging to A229 and A478 were genuine clones. There were 3, 5 and 2 off-types among the palms presumed to be from R291, R295 and A366 respectively, as indicated by 11, 11 and 16 numbers of SSR markers (Table 3.10).

"Off-types", where palms are wrongly labelled as a certain clone, occur in the field due to possible mistakes during tissue-culture or mix ups at nursery or during planting. Prior to field planting, oil palms go through extensive periods in tissue-culture and nursery. From a superior explant to culture and from cultures to nursery, it takes approximately 15 months and 27 months respectively. From nursery to the field, it takes another 18 months (Rohani and Tarmizi, 2003). Even with the best records, some numbers of mix-ups do happen.

The genotyping method, SSR based fingerprinting (Chee *et al*., 2015), was found to be an efficient and effective way of culling out the mix-ups. The 20 SSR primers selected exhibited good discriminatory power between clones. The results also clearly demonstrated the need for genotyping, to confirm the genetic identity of palms, for methodical comparisons within and between clones.

Incidentally all "off-types" were of normal phenotype and did not reduce the number of mantled palms available for the study. However, the plant resources had to be further reduced due to non-availability of normal and mantled palms of a clone in the same replicate, reversion of mantled palms, and variable severity in the mantled phenotype (selection round 3, figure 3.1). Other issues encountered in the field during the selection process included basal stem rot disease caused by *Ganoderma boninense* infection and early abortion or rotting of the inflorescences due to extreme weather conditions.

Table 3.10 Summary of Genotyping Results. The Genotyped samples are indicated in terms of palm number and phenotype (M= Mantled and N=Normal). Number of off types detected per clone and the primers that revealed these off types are summarised. Nos. means 'Numbers'.

Clone ID	Genotyped samples	Off types	Primers that indicated off-types
A229	64M, 67M, 69M, 76M, 77M, 68N, 70N, 71N, 72N, 73N (10 Nos.)	Zero	NA
R291	17M, 19M, 22M, 23M, 24M, 15N, 16N,18N, 20N, 21N, 1N, 2N, 3N, 4N, 5N (15 Nos.)	20N, 21N, 4N (3 Nos.)	sMg00025, sMg00042, sMg00108, mEgCIR00369, mEgCIR03428, mEgCIR03544, mEgCIR03808, mEgCIR02332, mEgCIR02492, mEgCIR06521, mEgCIR03298 (11Nos.)
R295	20M, 21M, 26M, 27M, 28M, 22N, 23N, 24N, 25N, 29N, 1N, 2N, 3N, 4N, 5N (15 Nos.)	22N, 23N, 25N, 29N, 4N (5 Nos.)	sMg00025, sMg00108, mEgCIR00369, mEgCIR03649, mEgCIR03544, mEgCIR03358, mEgCIR00783, mEgCIR03808, mEgCIR02332, mEgCIR03546, mEgCIR06521 (11Nos.)
A366	7551M, 7554M, 7558M, 7536N, 7550N, 7552N, 7553N, 7556N (8 Nos.)	7550N, 7556N (2 Nos.)	sMg00025, sMg00042, sMg00108, mEgCIR00369, mEgCIR03428, mEgCIR03649, mEgCIR02595, mEgCIR03358, mEgCIR03389, mEgCIR03808, mEgCIR03376, mEgCIR02332, mEgCIR02492, mEgCIR03311, mEgCIR02427, mEgCIR03546 (16Nos.)
A478	7839M, 7842M, 7838N, 7841N (4 Nos.)	Zero	NA

3.3.2. Phenotypic Characterisation of Fruit Bunches

Leaf stage from which the bunch was extracted, bunch weight, the number of spikelets sampled and the number of fruits sampled were recorded where possible during phenotyping (Table 3.11). Bunches extracted from young palms were smaller and lighter compared to bunches from mature palms. Fruit bunches collected from mature palms were from between leaf stages 19 and 24 (F19 to F24). They weighed on average 16.4 ± 3.85 kg. On the contrary, bunches harvested from young palms (sampling range F19 to F29) had an average weight of only 1.58 ± 0.53 kg. Thus, phenotyping of a selected subset of spikelets was done in the case of mature clones, twenty spikelets were selected from different parts of the bunches for visual scoring of fruits. Whole bunch phenotyping, where all the spikelets from the bunches were sampled and all the fruits were scored, was adapted for young clones.

Table 3.11 Summary of Bunch Characteristics. Range of sampling is expressed as leaf stage. Bunch weight, the number of spikelets and the number of fruits are expressed as mean ± standard deviation for each clone.

Clone	Number of Bunches Sampled	Range of Sampling	Bunch Weight (In kg)	Spikelets sampled	Fruits sampled
Mature Palms					
A229	2	19 to 19	-	-	225.00 ± 176.78
R291	4	23 to 23	14.50 ± 2.83	20.00 ± 0.00	260.50 ± 28.44
R295	1	24 to 24	20.20 ± NA	20.00 ± NA	393.00 ± NA
Young Palms					
A366	8	19 to 27	1.58 ± 0.53	41.38 ± 5.93	209.25 ± 66.59
A478	5	25 to 29	-	59.00 ± 6.96	341.80 ± 161.23

On average, 269.29 ± 94.17 fruits (from 20 spikelets) per bunch were scored in mature clones as opposed to 260.23 ± 125.53 fruits from 48.15 ± 10.78 spikelets per bunch (which was all the fruits in the bunch) scored in young clones. Multiple bunches were scored in the case of palms showing

a less severe or variable phenotype, and mean value of percentage mantled alongside standard deviation was used to represent its percentage abnormality (Table 3.12).

Previous publications did not refer to a standardised method for determining of mantled phenotype. In addition, the distinction between normal and mantled (often made through census data) is not enough for appropriate comparisons, as the mantled phenotype exhibits itself in varying degrees in the field. Though Jaligot *et al*. (2000, 2011) refers to slightly mantled and severely mantled palms, how this was determined or quantified was unclear.

Results were consistent with the sampling assumptions, all mantled palms belonging to mature clones were found to have a stable phenotype and were 100% mantled. Young, mantled palms not only had a lower mantled percentage but a high variability among the bunches sampled (Table 3.12). This was also in line with previous reports (Jaligot *et al*., 2011).

The scoring method explained here proved sufficient for conclusively determining the mantled status of the palms. The method is valid for both mature and young palms and provides a representative numerical value for the severity of mantled phenotype. The same may be used for tracking trends relating to reversion. Further examination of variability in homeotic transformation and fertility was done for more detailed characterisation of the mantled phenotype, details of which are included in chapter 4.

3.3.3. Results of *KARMA* Methylation Assay

The latest consensus on the origin of mantled abnormality is based on Ong-Abdullah *et al*. (2015, 2016). Altered methylation caused by the tissue-

culture process results in activation of a splice acceptor site situated in the transposable element now called "Mantled" that has homology to rice *Karma* LINE elements. This causes alternate splicing in the B class gene of oil palm *EgDEF1*, and accumulation of the truncated transcript during reproductive development. Thus Ong-Abdullah *et al*. (2015, 2018) proposed the *KARMA* methylation assay, which utilises differentially methylated regions (DMRs) for the early detection of the abnormal phenotype.

Methylation detection on the *Karma* transposon at the *EgDEF1* locus using *Rsa*I (Ong-Abdullah *et al*., 2015) conducted in technical triplicates and averaged for qPCR amplifications revealed three outliers (Table 3.12, Figure 3.5).

Table 3.12 Phenotyping Results and CHG Methylation Ratios of Mantled and Normal Palms. Phenotyping results based of visual scoring is expressed as percentage of abnormality/ percentage mantled (%M). Values are calculated means for each palm with standard deviation. CHG methylation ratios were calculated for *RsaI* restriction site of *EgDEF1 Karma* locus as described by Ong-Abdullah *et al*. (2015). Outliers are highlighted in green (false positive, a normal palm with low CHG methylation) and red (false negative, two mantled palms with high CHG methylation) font colours. %- Mean percentage value, SD- Standard Deviation

Clone/	Palm/	Phenotype	Mantled (% ± SD)	CHG methylation (%)
A229	69	Mantled	100.00 ±0.00	85
R295	21	Mantled	100.00 ±0.00	12
R291	17	Mantled	100.00 ±0.00	25
R291	19	Mantled	100.00 ±0.00	18
A366	7551	Mantled	53.03 ±22.60	56
A366	7554	Mantled	54.59 ±12.30	36
A366	7558	Mantled	43.46 ±33.97	21
A478	7842	Mantled	95.89 ±3.36	14
A478	7839	Mantled	66.65 ±36.66	20
A229	71	Normal	0.00	74
R291	18	Normal	0.00	77
R291	15	Normal	0.00	62
A366	7553	Normal	0.00	40
A366	7536	Normal	0.00	65
A478	7838	Normal	0.00	60
A478	7841	Normal	0.00	82

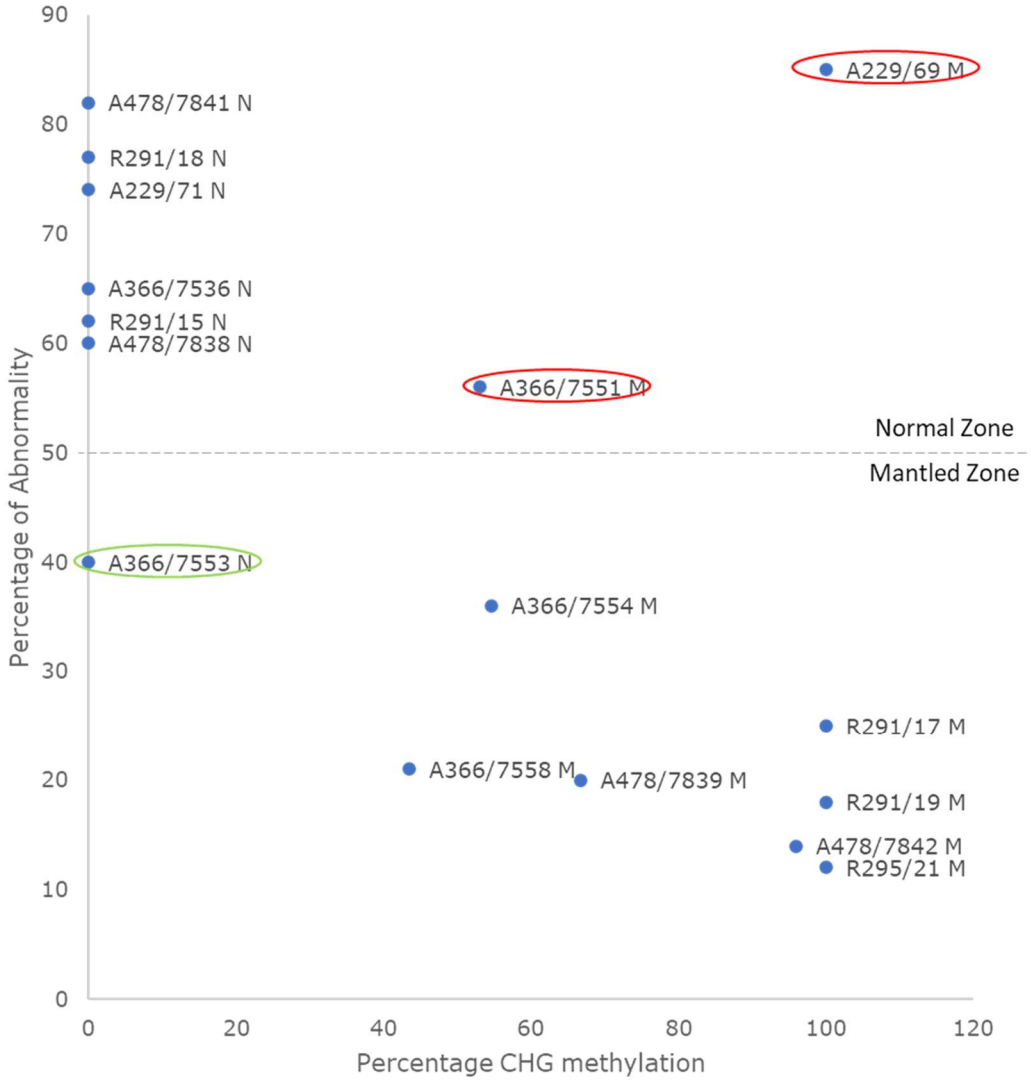

Figure 3.5 Assessment of the Effectiveness of *KARMA* Assay. Percentage CHG methylation at *RsaI* site of *EgDEF1 Karma* locus is plotted against the phenotyping score of Mantled and Normal samples. Data labels show palm ID in the format Clone-Palm Phenotype (M-Mantled, N- Normal). The outliers, a normal palm with low CHG methylation (false positive) and two mantled palms with high CHG methylation (false negative) are highlighted using green circle in red circles respectively.

It was expected that normal palms will have a high CHG methylation ratio whereas mantled palms will be hypomethylated and will have a low CHG methylation ratio. The palms A366-7551 M and A229-69 M phenotyped as mantled gave CHG methylation ratios of 85% and 56% respectively. Phenotyping results showed A366-7551 had a lower degree of mantled abnormality, that is 53.03±22.60% (average of 3 bunches) when sampled, whereas A229-69 M was fully mantled (100%).

The third outlier young clone A366/7553, which was phenotyped as normal had an unusually low CHG methylation ratio at the *EgDEF1 Karma*, while ratios of all other normal samples were consistent, ranging from 62%-82% (Table 3.12, Figure 3.5). This may be a false positive in the assay but, the possibility of this normal palm being a revertant may also be considered.

The testing here however, was limited to the *Rsa*I restriction site. Ong-Abdullah *et al*. (2015) carried out additional qPCR assays using *Bbv*I and *ScrFI* and supplementary bisulphite sequencing to analyse outliers. But these were not validated for the current set of samples. *Bbv*I qPCR assay was avoided due to possible SNP in the restriction site (Ong-Abdullah *et al*., 2015).

However, in the current sample set, the *KARMA* methylation assay shows 18.75% error (Table 3.12, Figure 3.5). This warrants further investigation. Similar errors in *KARMA* test were pointed out by Weckx *et al*. (2019) as well, and this upholds the necessity for morphological characterisation. Results suggest combined use of multiple detection techniques, morphological and/or molecular, reduces errors in determination of mantled phenotype.

3.4. SUMMARY

Identification of comparable normal and mantled ramets belonging to different clones was achieved through molecular and morphological characterisation of ramets. Available plant material was screened to eliminate unstable phenotypes and environmental influences on the results. Accurate determination of genotypic identity was achieved through genotyping using SSR markers. Twenty SSRs were used to develop unique fingerprints for 52 palms belonging to the 5 different clones. The SSRs exhibited good discriminatory power between clones. Using the results 10 "off-types", that is wrongly labelled ramets in the field, not belonging to said clones, were identified and excluded from sampling. So, the genotyping method, was found an efficient and effective way of culling out the off-types.

Phenotypic identity or the mantled status of the ramets were determined by morphological characterisation via phenotyping of unripe fruit bunches and *KARMA* assay. As no previous publications had described a standardised method for quantifying mantled severity, a new phenotyping protocol was designed. Phenotyping was done by meticulous scoring of individual fruits of the entire bunch or 20 selected spikelets in the case of young palms and mature palms respectively. The mantled percentage thus calculated denotes the proportion of mantled fruits in the unripe fruit bunch(es) sampled. Mantled percentage proved useful for comparisons.

The *KARMA* assay however showed 18.75% error in the current sample set. This included two false positives and one false negative. Two of the mantled palms showed a high percentage of CHG methylation while one of the normal palms showed low methylation percentage. This warrants further investigation.

CHAPTER 4
CHARACTERISATION OF MANTLED FRUIT BUNCHES

4.1. INTRODUCTION

Objectives:

- Detailed phenotypic characterisation of mantled fruit bunches to account for the heterogeneity in homeotic transformation and fertility associated with the phenotype.

Oil palm crop is inherently heterogeneous. Even though palms belonging to a clone are in theory genetically identical, at the field level, significant differences are observed between the individual palms, as a consequence of epigenetic changes and environmental influences. Mantled abnormality, in particular, is known to occur at varying degrees in palms belonging to the same clone. The occurrence and severity of mantled abnormality have been reported to be highly "unpredictable" (Jaligot *et al*., 2011).

The abnormal mantled phenotype manifests in fruit bunches as distinctive "mantled fruits", characterised by the presence of pseudocarpels. Pseudocarpels are formed by the homeotic transformation of staminodes in female flowers. It is also noted that in severely mantled palms, the main carpel is sterile. Molecular events leading to sterility are unclear (Ooi *et al*., 2019). While previous studies mention these characteristics of the mantled phenotype, none have quantified them or explored their relationship with the severity of the abnormality in detail.

Mantled phenotype exhibits "Spatial and temporal heterogeneity" (Jaligot *et al*, 2002). Further differences in severity of mantled have been shown to result in different protein profiles (Yaacob *et al*., 2013). So, to correctly assess the phenotype of the samples and to enable meaningful comparisons

between and within clones, thorough phenotyping and characterisation of the research material were necessary.

Ordinarily, characterisation and grading of oil palm bunches are done to ascertain a ripeness level for maximum oil yield (Alfatni *et al*., 2013). However, since the "mantled" phenotype is deleterious when identified, the palms are culled from commercial planting, hence the bunches are seldom characterised or graded. So, there were no standardised grading methods available for assessing mantled severity and variability across mantled fruit bunches. However, for the purpose of studying this phenotype, proper characterisation of mantled bunches was required.

Thus, the phenotype of sampled palms was analysed by meticulous visual scoring of unripe bunches, to formulate a phenotyping regime and identify comparative indices. This phenotyping regime was differentiated for small fruit bunches of young and big fruit bunches of mature ramets. Data collected were compared against fruit bunches from normal palms of the same clone and replicate.

The genotypic and phenotypic identities of all ramets used in the study were determined as described in chapter 3. Mantled percentage (%M) was calculated for each ramet to establish its mantled phenotypic identity. But mantled percentage alone could not embody the full picture. Mantled percentage illustrates percentage abnormality which reflects the severity of the mantled phenotype but not the variability. It was proposed that by calculating parameters concerning homeotic transformation and fertility of fruits and analysing their distribution, the composition of bunches and variability of phenotype may be represented scientifically. Accordingly, a mathematical approach to analyse mantled fruit bunches for the severity and variability of phenotype is discussed here.

4.2. MATERIALS & METHODS

See chapter 3, sections 3.2.1 and 3.2.9 for details of plant material and method of bunch selection and sampling respectively.

4.2.1. Phenotypic Characterisation of Fruit Bunches

Phenotypic characterisation of fruit bunches was done through visual assessment and scoring of all fruits in the bunch, in the case of young palms, and fruits from selected 20 spikelets in the case of mature palms (See section 3.2.9). Phenotype, number of pseudocarpels and fertility status of individual fruits were recorded (Figure 4.1). Fruits were characterised as Normal when there was no pseudocarpels present. The presence of a developing kernel was used as the determinant of fertility (Figure 4.1). In the case of mantled fruits, fruits were categorised as Normal or PC_1 to PC_8 based on the number of pseudocarpels present (Figure 4.1).

The data thus generated was used to calculate phenotyping parameters for the bunch. Mantled percentage (%M) was calculated as detailed in section 3.2.9.

Supplementary phenotyping parameters were calculated as follows:

Percentage Fertility in Mantled (%FM) $= \frac{number\ of\ fertile\ mantled\ fruits\ (F)}{number\ of\ mantled\ fruits\ (M)}$

Where F is the number of fruits with a developing kernel (fertile) showing homeotic transformation (mantled) and M is the total number of fruits showing homeotic transformation (mantled).

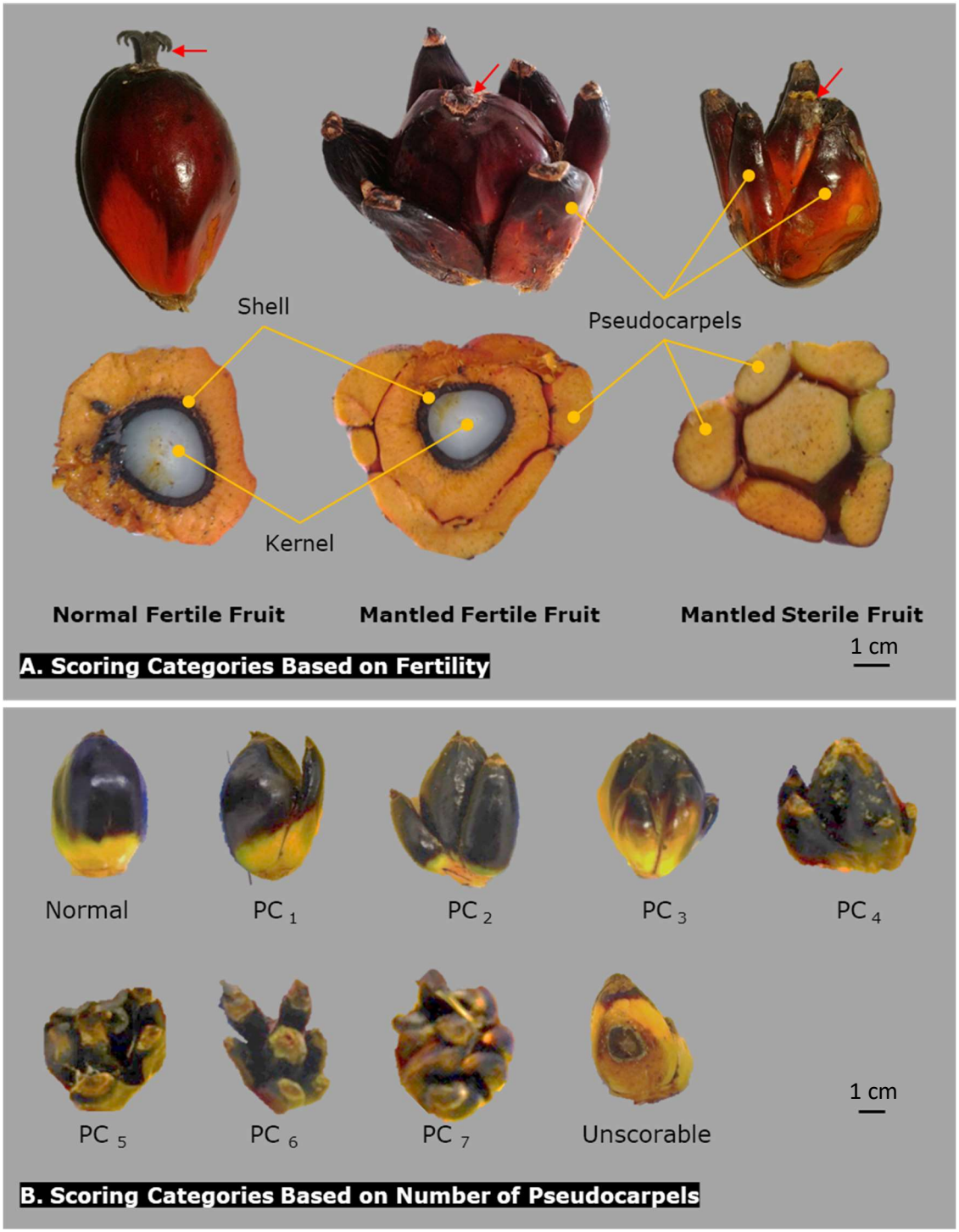

Figure 4.1 Criteria for Visual Scoring of Individual Fruits. Morphological phenotyping of fruits, bunches and palms was achieved through (A) visual scoring based on the presence or absence of pseudocarpels and a developing/developed kernel. Further classification of mantled fruits into scoring categories PC_1 to PC_7 (PC refers to pseudocarpels) was done based on the number of pseudocarpels, seen as fleshy finger-like projections around the carpel(B). Damaged fruits whose phenotype was not scorable were categorised as "unscorable". Red arrows shows necrotic stigma that has broken off from most fruits. The colour difference in A and B are due to the difference in ripeness, fruits in A are ripe (close to harvest maturity) and B are unripe.

Percentage Sterility in Normal (%SN) $= \frac{number\ of\ sterile\ normal\ fruits\ (S)}{number\ of\ normal\ fruits\ (N)}$

Where S is the number of normal fruits without a developing kernel (sterile) and N is the total number of normal fruits (no homeotic transformation).

Mode of the Number of Pseudocarpels in all Fruits (PC Mode$_{0\text{-}8}$) = *Most frequently appearing number of pseudocarpels in the fruit bunch.*

In the case of PC Mode$_{0\text{-}8,}$ all the fruits, both normal and mantled, in mantled bunches were taken into account.

Mode of the Number of Pseudocarpels in Mantled Fruits (PC Mode$_{1\text{-}8}$) = *Most frequently appearing number of pseudocarpels among the mantled fruits in the fruit bunch.*

In the case of PC Mode$_{1\text{-}8,}$ the normal fruits (without pseudocarpels) in mantled bunches were disregarded.

Weighted Mean of the Number of Pseudocarpels (PC Mean$_{wt}$) $= \frac{\Sigma WX}{\Sigma X}$

Where W is the number of pseudocarpels (0 to 8) and X is the number of fruits recorded with said number of pseudocarpels.

Each parameter was calculated for a fruit bunch. Accordingly, the parameters of a palm were calculated by finding the average of all bunches scored. Mean values were supplemented with standard deviation where applicable.

Standard Deviation (σ) = $\sqrt{\frac{\Sigma(X-\mu)^2}{n}}$

Where X is the value of observation, μ is mean and n is the number of observations.

4.2.2. Statistical Analysis

All statistical analyses of data were carried out using Genstat 64-bit Release 18.2 (PC/Windows 8) Copyright 2016, VSN International Ltd. Model checking was done by plotting the residuals against the standardised residuals for normal distribution. Regression analysis using generalised linear model was used unless otherwise specified.

Binomial distribution (binomial total 100) was adapted for the analysis of percentage data. Logit transformation was used for stabilizing the variance of percentage data as follows:

$$\boldsymbol{Logit(p)} = \log\left(\frac{p}{1-p}\right)$$

Where p is the proportion, and the log is the logarithm to the base e.

4.3. RESULTS AND DISCUSSION

Phenotyping data was used to assess the severity and variability of mantled phenotype. Fruit bunches were evaluated not only in terms of the percentage of fruits showing mantled abnormality (%M) but also with respect to the degree of homeotic transformation and sterility of individual fruits.

Three phenotyping parameters were calculated to numerically express the degree of homeotic transformation namely, the mode of the number of pseudocarpels among all fruits (PC $Mode_{0-8}$), the mode of the number of pseudocarpels among mantled fruits (PC $Mode_{1-8}$) and the weighted mean of the number of pseudocarpels (PC $Mean_{wt}$). The fertility status of mantled and normal fruits in the bunched was calculated separately and was expressed as the percentage fertility among mantled fruits (%FM) and the percentage sterility among normal fruits (%SN; Table 4.1). The full phenotyping data is included in appendix 3.

Bunches from all normal palms included in the study scored 0% mantled, that is all fruits scored were normal, and showed 0% sterility. Mature mantled palms (A229/69, R291/17 and R291/19) were 100% mantled, in other words, all fruits scored showed homeotic transformation, that is presence of pseudocarpels.

Young mantled palms had varying degrees of mantled percentage and fertility. In addition, there was high variability between fruit bunches from the same palm, as indicated by the standard deviations associated with %M, %FM and %SN (Table 4.1).

Table 4.1 Phenotyping Result Summary. Palm ID is in the format Clone/Palm Phenotype, where M- Mantled, N- Normal. All values shown are the average of the bunches scored per palm. Percentage abnormality (%M), fertility in mantled (%FM) and sterility in normal (%SN) are expressed in percentage mean ± standard deviation. PC Mode is the most frequently occurring number of pseudocarpels (in the ranges specified) among the fruits of the bunches scored. PC $Mean_{wt}$ is the weighted mean of the number of pseudocarpels in the bunches.

Palm ID	Bunches Scored	%M	%FM	%SN	PC $Mode_{0-8}$	PC $Mode_{1-8}$	PC $Mean_{wt}$
Mature Palms							
A229/71 N	1	0.00	0.00	0.00	0	NA	NA
R291/15 N	1	0.00	0.00	0.00	0	NA	NA
R291/18 N	1	0.00	0.00	0.00	0	NA	NA
R295/24 N	1	0.00	0.00	0.00	0	NA	NA
A229/69 M	1	100.00	0.00	0.00	5	5	5.18
R291/17 M	1	100.00	0.00	0.00	6	6	5.59
R291/19 M	1	100.00	3.62	0.00	6	6	4.97
Young Palms							
A366/7553 N	1	0.00	0.00	0.00	0	NA	NA
A478/7838 N	1	0.00	0.00	0.00	0	NA	NA
A366/7558 M	2	43.46 ± 33.96	31.65 ± 44.77	0.00 ± 0.00	0	2	1.66
A366/7551 M	3	53.03 ± 22.60	88.68 ± 19.61	0.00 ± 0.00	0	1	1.96
A366/7554 M	2	54.59 ± 12.30	33.50 ± 47.38	11.21 ± 15.85	0	1	1.73
A478/7839 M	2	66.65 ± 36.66	31.03 ± 19.74	47.02 ± 10.93	0	1	3.04
A478/7842 M	2	95.88 ± 3.36	10.01 ± 12.20	26.67 ± 9.43	6	6	4.92

4.3.1. Variability in Homeotic Transformation

The number of pseudocarpels per fruit specified the degree of homeotic transformation (Figure 4.1). The number of pseudocarpels per mantled fruit varied from zero (normal fruit) to 8. While six pseudocarpels may be accounted for by the homeotic transformation of abortive staminodes in the female flower, the origin of the seventh and eighth pseudocarpels is not clear. The percentage of fruits with each number of pseudocarpels (0 to 8) scored among bunches from different palms are shown in Figure 4.2. The statistical mode was calculated to reflect the most frequently occurring

number of pseudocarpels among all fruits (PC $Mode_{0-8}$), and among mantled fruits (PC $Mean_{wt}$) (Table 4.1).

Mature mantled palms (A229/69, R291/17 and R291/19) were 100% mantled and had a PC $Mean_{wt}$ greater than 4 indicating a higher degree of homeotic transformation. Most mantled fruits scored had 6 pseudocarpels except in the case of A229/69 wherein most mantled fruits scored had 5 pseudocarpels (Table 4.1).

Mantled palms belonging to the young clone A366 showed a similar pattern in the proportion of fruits with each number of pseudocarpels (Figure 4.2). Bunches from all three mantled palms had a very low degree of homeotic transformation (PC $Mean_{wt}$ <2) with most mantled fruits having only 1 or 2 pseudocarpels (PC $Mode_{1-8}$ being 1 for 7551 and 7554 and 2 for 7558).

Young clone A478 had highly variable results between palms. Palm 7839 had a low mantled percentage (66.65 ± 36.66), a lower degree of homeotic transformation (PC $Mean_{wt}$ of 3.04), and most mantled fruits had only 1 pseudocarpel. Whereas A478-7842 young palm with the highest mantled percentage showed a very similar pattern to mature 100% mantled palms with respect to homeotic transformation.

The variation in the number of fruits bearing each number of pseudocarpels in the case of 100% mantled palms of mature clones (Figure 4.3) and less severely mantled palms of younger clones (Figure 4.4) were examined alongside 100% normal palms belonging to the same clones.

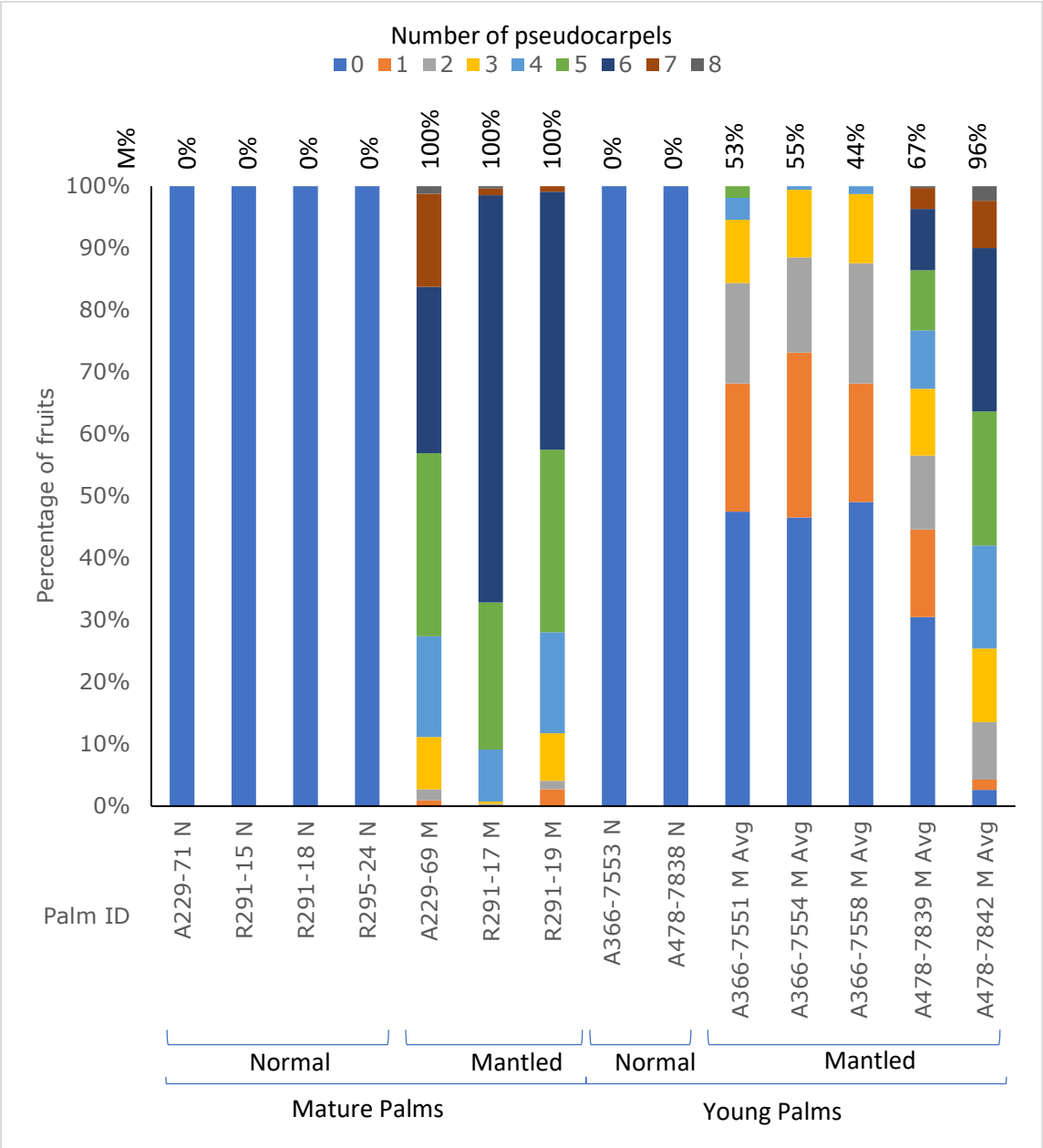

Figure 4.2 Percentage Distribution of Homeotic Transformation across Fruit Bunches. Percentage of fruits belonging to each category based on the number of pseudocarpels (0 to 8 as indicated by the different colours) in 4 normal and 3 mantled mature palms and 2 normal and 5 mantled young palms are presented. The percentage of abnormality (%M) of each palm is given on top. Palm ID at the bottom, in the format Clone-Palm Phenotype. M-Mantled, N-Normal. Avg- Average Data

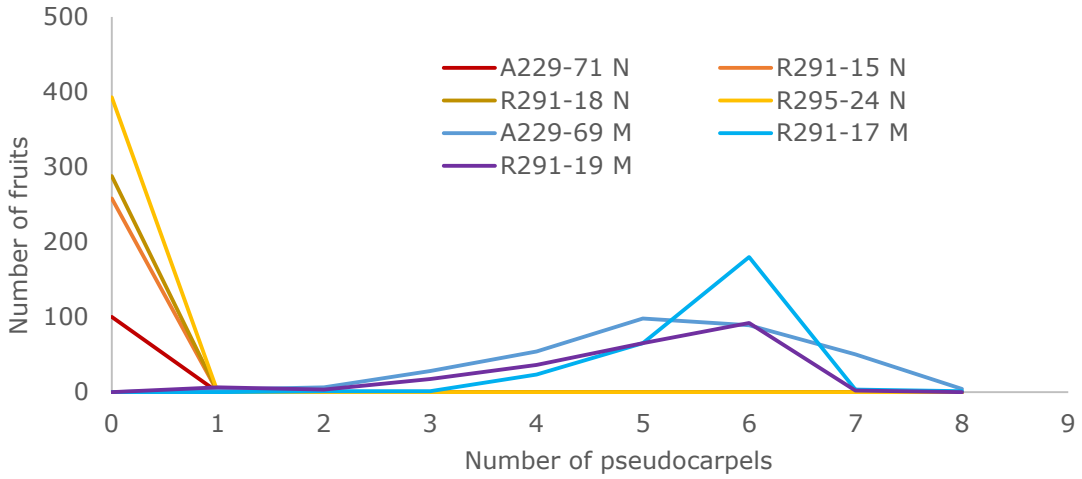

Figure 4.3 Variability in the Number of Pseudocarpels per Fruit across Fruit Bunches from Mature Palms.

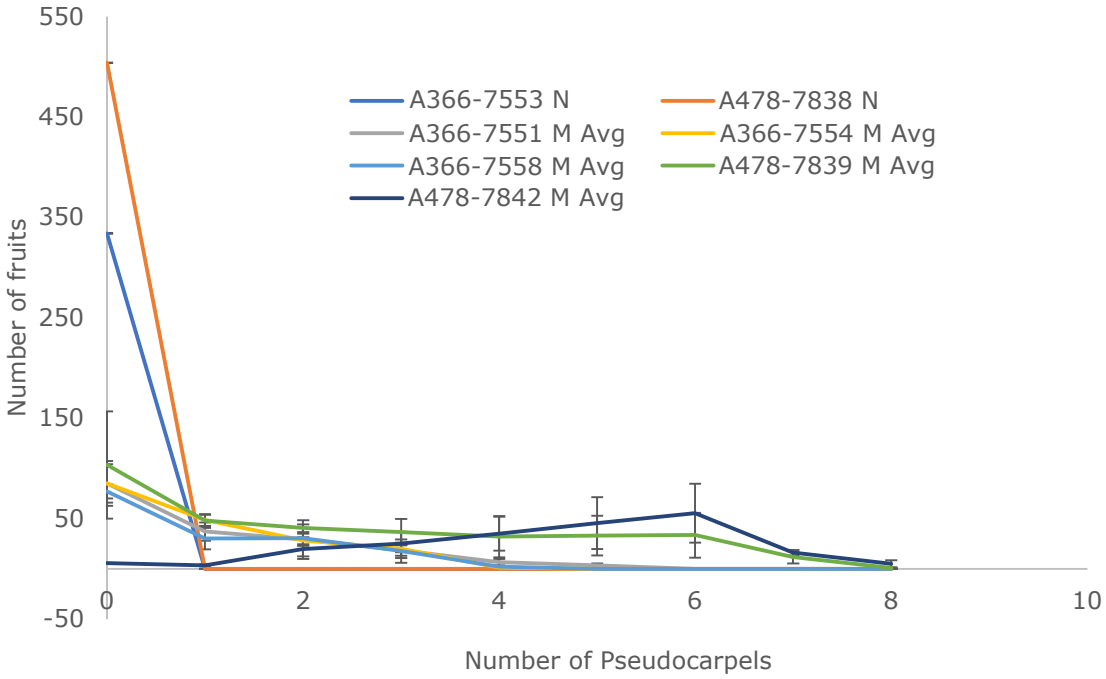

Figure 4.4 Variability in the Number of Pseudocarpels per Fruit across Fruit Bunches from Young Palms. The average of multiple fruit bunches has been calculated to reflect bunch characteristics of individual palms, and the dispersion of data is indicated by error bars showing standard deviation.

In the case of 100% mantled mature palms R291-17, R291-19 and severely mantled young palm A478-7842 (95.88±3.36% Mantled) most fruits carried six pseudocarpels (Table 4.1, Figure 4.2), showing the homeotic transformation of all six abortive staminodes. Even though 100% mantled mature palm A229-69 had PC $Mode_{0\text{-}8}$ value 5, that is most mantled fruits scored had five pseudocarpels instead of six, it had a PC $Mean_{wt}$ greater than

5. Further examination shows 98 fruits scored had five pseudocarpels, and 89 had six (Figure 4.3). So, while Mode values gave a good indication of the severity of homeotic transformation, by representing the number of pseudocarpels in the largest scoring group, PC $Mean_{wt}$ provided a clearer picture of the degree of transformation. PC $Mean_{wt}$ was especially useful when the scoring groups were very close together in numbers as was the case with A229-69.

Palms belonging to clone A366 namely 7551 (53.03±22.60% Mantled), 7554 (54.59±12.30% Mantled) and 7558 (43.46±33.96% Mantled) had the highest number of fruits (84%, 89% and 88% respectively) with zero to two pseudocarpels. Palm 7839 belonging to clone A478 with a mantled percentage of 66.65±36.66 had 56.55% fruits with zero to two pseudocarpels (Figure 4.1). This is indicative of a lower degree of homeotic transformation and is reflected in their PC $Mean_{wt}$.

Overall, the results confirmed the assumption that mantled phenotype is less stable among young ramets. However, similarity in phenotypic expression patterns within the clones R291 and A366 may be an indication of genetic effects. If the similar mantled status of the palms within these clones is due to the genetic resilience of the clone, the same could be the reason for the number of pseudocarpels or homeotic transformations observed. It is noteworthy that none of the young palms showed a steady decreasing mantled percentage (or degree of homeotic transformation) towards younger bunches scored (Appendix 3) which would be expected if the palm was reverting. In fact, the data appeared random, and it was not possible to make a definite inference on the reversion of palms from the limited sample set.

As for the young clone A478, if environmental and genetic effects may be excluded as samples were taken from the same replicate, and the genetic identity of palms were confirmed, a likely reason for within clone variances is epigenetic differences within the clone. Jaligot *et al* (2002) has previously suggested that "spatial and temporal heterogeneity" of the mantled phenotype is due to an epigenetic origin. Further, the current consensus on the origin of the phenotype refers to differential methylation of *EgDEF*, which may be epigenetically reformed (Ong-Abdullah *et al*., 2015). As clones (even of the same genotype) may come from different explants, there may be tissue-culture induced differences that persist, which could be influenced by the environment. Whether a bunch fully expresses the genetic/epigenetic marks, could also be influenced by environmental factors (Smulders and De Klerk, 2011). But the validity of the results is limited by the small sample size. However, the phenotyping regime and parameters described are useful and effective for the analysis of variability of homeotic transformation in mantled palms, between and within clones.

4.3.2. Correlation between Percentage Abnormality and Degree of Homeotic Transformation

The weighted mean of the number of pseudocarpels was used as a numerical indicator of the extent of homeotic transformation across the bunch. On statistical analysis, it was found to be strongly correlated with the percentage abnormality of the bunch with a correlation coefficient of 0.92 (Figure 4.5). The correlation is positive however, the relationship may not be linear (Figure 4.5). It is evident that a lower degree of homeotic transformation is closely linked to the lower severity of the abnormality.

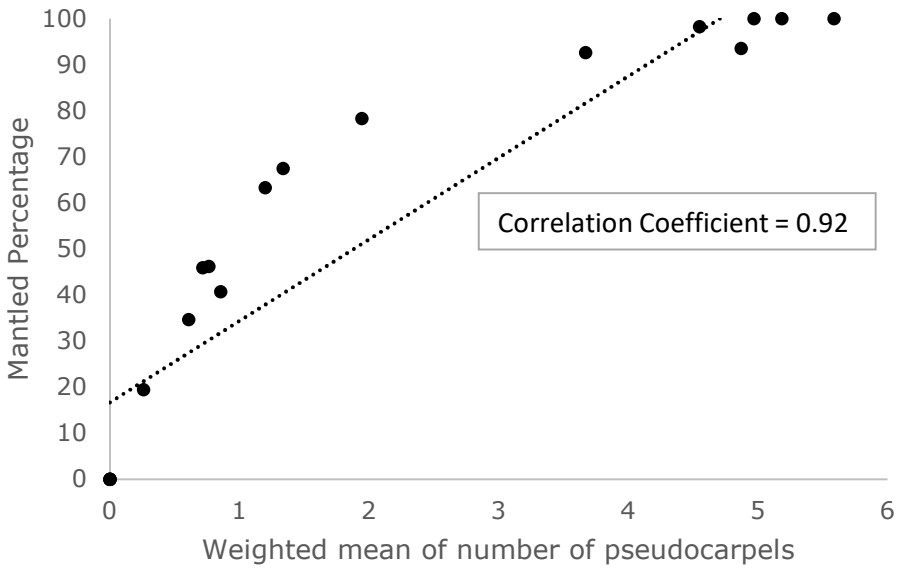

Figure 4.5 Correlation between PC Mean$_{wt}$ and %M. The scatter plot and trend line show a strong positive correlation between the weighted mean of the number of pseudocarpels (PC Mean$_{wt}$) and the percentage of abnormality (%M) of bunches. Data indicates a positive non-linear relationship with a correlation coefficient of 0.92 between the two parameters.

4.3.3. Variability of Fertility in Mantled Bunches

The fertility of fruit was recorded alongside the number of pseudocarpels during phenotyping. For each fruit bunch scored the percentage fertility in each category (PC 0 to 8) was calculated in addition to the percentage fertility of all mantled fruits and the percentage sterility of all normal fruits. In the case of young palms that displayed lower severity of abnormality as well as high variability between bunches, 2 to 3 bunches were scored, and the average values were considered along with the standard deviation. (Table 4.2, Figure 4.6).

All normal palms had 100 per cent normal (PC 0) fertile fruits. Mature 100% mantled palms A229-69 and R291-17 exhibited complete sterility in all categories (PC 1 to 8).

Interestingly R291-19 showed 3.62% fertility among mantled fruits (Table 4.1, 4.3). According to previous reports, the sterility of fruit relates to the severity of mantled phenotype (Beulé *et al*., 2010; Ong-Abdullah *et al*.,

2015). In less severely mantled palms, the carpel is usually fertile, but in severely mantled palms, they are parthenocarpic and sterile. It is fascinating to note that this was also a palm that recorded one of the lowest CHG methylation percentages (18%) in the sample set (see section 3.3.3). In the case of R291-19, the percentage fertility decreased with the increase in the number of pseudocarpels, that is higher fertility was observed in fruits with a lower number of pseudocarpels signifying a lower level of homeotic transformation. While the palm showed 2.17% fertility in fruits with six pseudocarpels, 33.33% of fruits with only a single pseudocarpel were fertile.

A similar pattern is observed among the young clones as well; maximum fertility was observed in the normal fruits of the mantled bunches followed by fruits with a lower number of pseudocarpels or lower degree of homeotic transformation. In A478-7842 which had an average of 10.01 ± 12.20% fertility among the total mantled fruits, 73.33% fertility was observed in normal fruits, followed by 20% and 15% in fruits with one and two pseudocarpels respectively.

Table 4.2 Percentage Fertility of Fruits with Pseudocarpels 0 to 8. Palm ID is in the format Clone/Palm Phenotype, where M- Mantled, N- Normal. All values shown are the average of the bunches scored per palm. Categories PC 0 to 8 are based on the number of pseudocarpels observed. Percentage fertility is stated as NA where there were no fruits scored in that category. Therefore 0 percentage fertility indicates all fruits scored in the category are sterile/parthenocarpic. PC $Mean_{wt}$ is the weighted mean of the number of pseudocarpels in the bunches. Percentage abnormality (%M), fertility in mantled (%FM) and sterility in normal (%SN) are expressed in percentage mean ± standard deviation.

Palm ID	PC $Mean_{wt}$	%M	Percentage Fertility of Fruits									%FM	%SN
			Normal	Mantled									
			PC 0	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8		
Mature Palms													
A229/71 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
R291/15 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
R291/18 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
R295/24 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
A229/69 M	5.18	100.00	NA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
R291/17 M	5.59	100.00	NA	NA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
R291/19 M	4.97	100.00	NA	33.33	0.00	5.88	2.78	3.08	2.17	0.00	NA	3.62	0.00
Young Palms													
A366/7553 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
A478/7838 N	NA	0.00	100.00	NA	NA	NA	NA	NA	NA	NA	NA	0.00	0.00
A366/7558 M	1.66	43.46 ± 33.96	100.00	37.78	28.57	32.35	0.00	NA	NA	NA	NA	31.65 ± 44.77	0.00
A366/7551 M	1.96	53.03 ± 22.60	100.00	83.33	94.12	100.00	66.67	66.67	NA	NA	NA	88.68 ± 19.61	0.00
A366/7554 M	1.73	54.59 ± 12.30	88.79	32.50	34.85	34.00	50.00	NA	NA	NA	NA	33.50 ± 47.38	11.21 ± 15.85
A478/7839 M	3.04	66.65 ± 36.66	52.98	45.71	34.38	11.82	17.21	7.38	47.69	0.00	0.00	31.03 ± 19.74	47.02 ± 10.93
A478/7842 M	4.92	95.88 ± 3.36	73.33	20.00	15.15	10.71	14.41	9.76	4.12	6.67	0.00	10.01 ± 12.20	26.67 ± 9.43

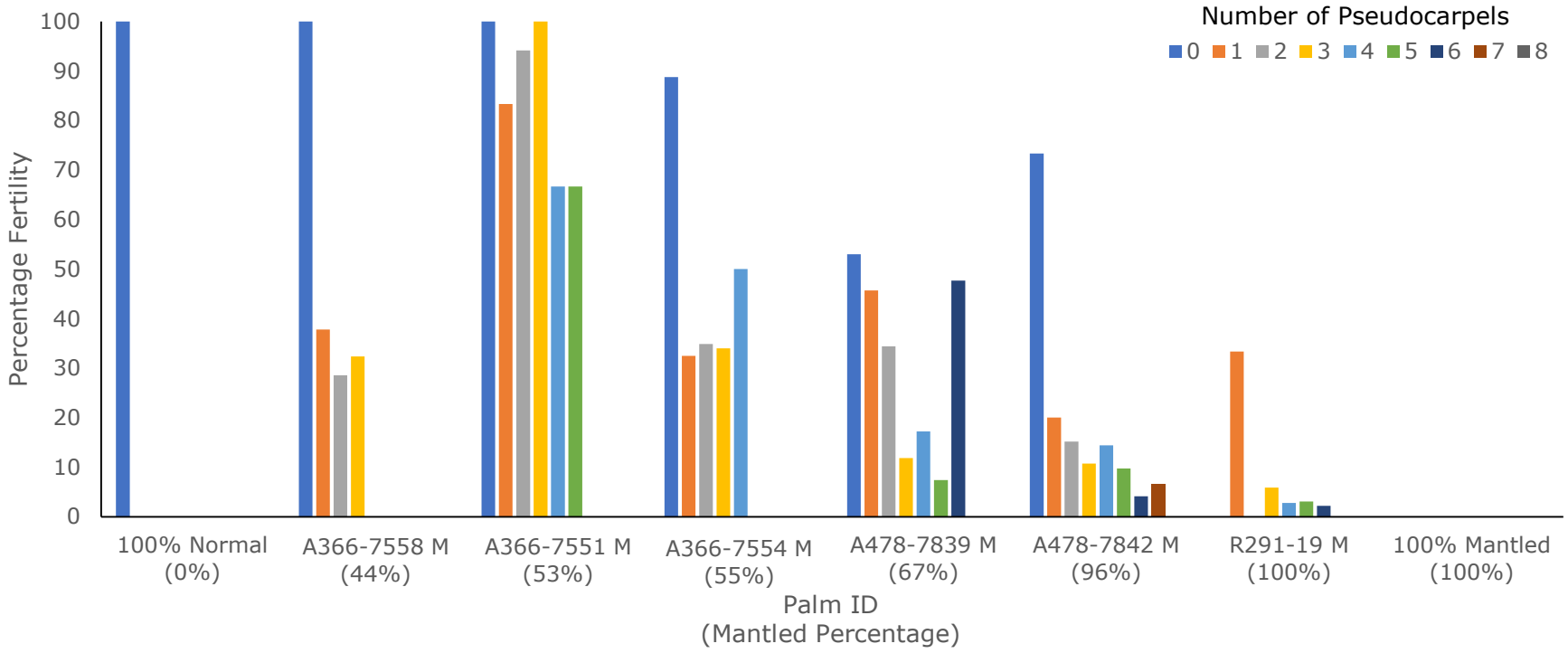

Figure 4.6 Percentage Distribution of Fertility of Fruits with 0 to 8 Pseudocarpels. Data is shown from least mantled to most mantled palms phenotyped. The axis label '100% Normal' represents all normal palms (no fruits scored in categories 1 to 8) that are A229-71N, R291-15N, R291-18N, R295-24N, A366-7553N and A478-7838N. The axis label '100% Mantled' represents all 100% mantled palms, with no fertile fruits that are A229-69 and R291-17. Palm ID in the format "Clone-Palm Phenotype", M- Mantled, N- Normal. Mantled percentage included in brackets shows Percentage of abnormality.

In the case of A478-7839 (fertility in total mantled fruits 31.03 ± 19.74%) percentage of fertility were 52.98%, 45.71% and 34.38% in categories pseudocarpels 0 (normal), 1 and 2, respectively. But fruits with 6 pseudocarpels also showed a higher percentage of fertility (47.69%), breaking the pattern. Further palm 7839 had a low mantled percentage (66.65 ± 36.66) in comparison but higher percentage sterility (47.02 ± 10.93) among its normal fruits. However, there was a large variation between bunches from the same palm (Table 4.2).

In clone A366, bunches from palms 7558 and 7551 showed 100% fertility in normal fruits. In fact, 7551 had 88.68 ±19.61% fertility in mantled fruits as well. Interestingly the percentage fertility of 7558 was only 31.65 ± 44.77% even though it had a lower percentage of abnormality. However, bunches phenotyped from both 7558 and 7554 were very different from one another in terms of percentage of abnormality as well as fertility, hence their indices have large standard deviations attached to them.

Data indicated a high amount of variability in fertility between bunches of the same palm, as well as between mantled fruits from the same bunch. But as a general trend percentage of fertility among mantled fruits decreased with an increase in mantled severity, and this inverse relationship has a negative correlation coefficient of -0.77 (Figure 4.7).

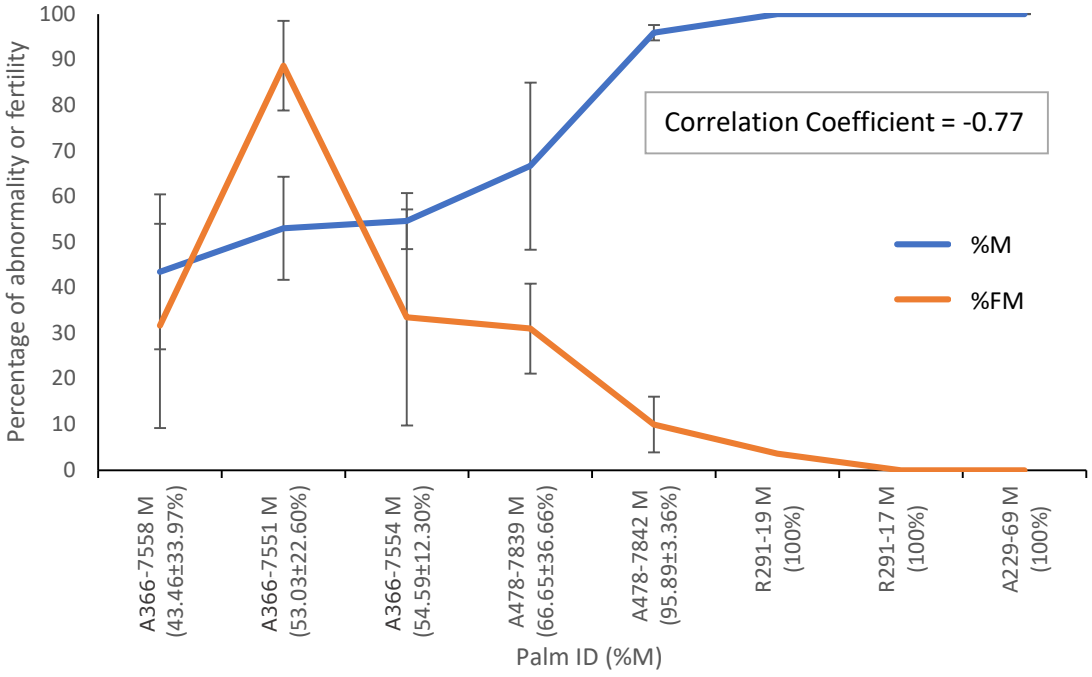

Figure 4.7 Correlation between %M and %FM. The percentage of abnormality (M%, blue line) and the percentage fertility in mantled fruits (%FM, orange line) are shown for each mantled palm that was phenotyped by visual scoring. Data is shown from least mantled to most mantled palms. Palm ID is in the format clone-palm and phenotype (M for mantled). The mean mantled percentage of palms with standard deviation are is also shown within the bracket. The error bars indicate the standard deviation associated with mean values of %M and %FM. A lower standard deviation was associated with mature clones R291 and A229. A strong negative correlation with a Correlation coefficient of -0.77 is observed between the two parameters.

Young mantled palm, A366-7551 showed a high percentage of fertility despite a relatively high mantled percentage, a tendency that was not apparent in other palms of the same clone. The reason for this unusual drift is unknown. While the overall association between fertility in mantled fruit and severity of the phenotype is clear from the current data, a larger sample set is needed for further examination of this relationship.

4.4. SUMMARY

Mantled phenotype is highly varied in its expression. Large variabilities exist not only in the severity of mantled phenotype but also in the degree of homeotic transformation and fertility of fruits in the mantled bunches. A phenotyping regime, based on visual scoring, was developed for the detailed characterisation of mantled fruit bunches. The phenotyping regime enables precise characterisation of mantled severity with respect to the extent of homeotic transformation and fertility of fruits.

Visual scoring was done for multiple whole fruit bunches in the case of young palms. Since scoring all the fruits per bunch was a herculean task in the case of mature palms the method was adapted for them to include only selected spikelets (Sections 3.2.1). The scoring data was further analysed to yield specific parameters that reflect on severity and variability of the abnormality namely, mantled percentage (%M), percentage fertility in mantled fruits (%FM), percentage sterility in normal fruits (%SN), mode of the number of pseudocarpels for all fruits (PC $Mode_{0\text{-}8}$) and mantled fruits (PC $Mode_{1\text{-}8}$) and finally the weighted mean of the number of pseudocarpels *(PC* $Mean_{wt}$*)*. The percentage distribution of fruits with different numbers of pseudocarpels and fertility across these categories was also calculated.

The weighted mean of the number of pseudocarpels was found to be highly correlated to the percentage of abnormality meaning as the severity of abnormality increases, so does the extent of homeotic transformation/number of pseudocarpels per fruit. As expected, fertility in fruits decreased with the severity of mantled, but fertility was found to be highly variable within bunches and also between bunches of the same palm.

A more severe and stable mantled phenotype was observed in mantled mature palms. They bore 100% mantled fruits with a higher degree of homeotic transformation. The mantled fruits were mostly sterile if not all. Hence mature palms of 10 years of age were found well suited for comparative studies between normal and mantled phenotypes.

However, in young palms, the mantled phenotype was found to be highly dynamic. In general, a lower mantled percentage was associated with a lesser degree of homeotic transformation and higher fertility in normal and mantled fruits. But the results were varied for different fruit bunches from the same palms (temporal heterogeneity) and across the same fruit bunch (spatial heterogeneity). A478-7842 young palm with the highest mantled percentage in the set showed a very similar pattern to mature 100% mantled palms with respect to homeotic transformation but differed with respect to the fertility in mantled fruits and sterility in normal fruits. Overall, the results confirmed the assumption that the mantled phenotype is less stable among young ramets.

Nevertheless, the phenotyping regime and mathematical parameters proposed may be used to account for this heterogeneity and phenotypic complexity of mantled phenotypes.

CHAPTER 5
OIL PALM INFLORESCENCE SAMPLING AND HISTOLOGY

5.1. INTRODUCTION

Objectives:

- Optimisation of inflorescence sampling protocols for the non-destructive and destructive sampling range, including evaluation of the efficacy of inflorescence length as a field reference for the inflorescence stage.
- Comparison of different fixatives available to optimise microscopy protocol for oil palm inflorescence samples.

Oil palm flowers are perhaps one of the most protected flowers in the plant kingdom, making them difficult to get to for detailed studies or monitoring. Studying oil palm flower development requires sampling of inflorescences at the base of fronds embedded within the tree trunk. Individual oil palm inflorescences develop over two years of time, in the axils of subtending fronds, protected within the leaf base (Figure 5.1). The earlier, younger developmental stages cannot be reached without damaging the meristem or killing the palm (destructive sampling).

The study of reproductive development in oil palm is challenging in various respects, but it is not new. Thirty years ago, Van Heel *et al*. (1987) studied oil palm reproductive development using scanning electron microscopy

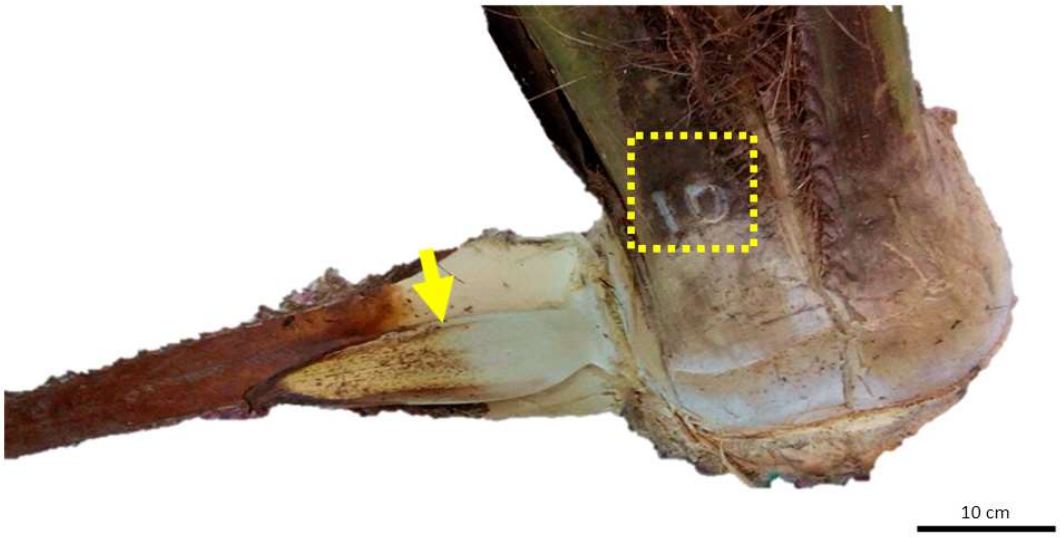

Figure 5.1 Young Inflorescences at the Base of Subtending Leaf. The inflorescence indicated by the arrow is still covered in spathes. Numbering on the younger frond visible (dotted box).

(SEM) and gave a detailed description of early inflorescence and floral development. The most comprehensive study on the subject is, of course, Adam *et al*. (2005), where the reproductive development complexities of oil palm from inflorescence initiation were described using light and scanning electron microscopy.

For a systematic and thorough examination of developing inflorescences of oil palm across the developmental stages, a standardised sampling and histology protocol was necessary. The protocols were adapted from previous literature and communications with MPOB and AAR teams (Adam *et al*., 2005, Sarpan *et al*., 2015). Optimisation of the protocols was done by trials as described here. Initial assessments were made using a reference series prepared by selective sampling. The range of dimensions of samples attainable from the sampling range was surveyed to fine-tune field procedures. A comparison was made between two different fixation buffers previously used by MPOB and CIRAD (Adam *et al*., 2005, Sarpan *et al*., 2015), namely glutaraldehyde–paraformaldehyde–caffeine (GPC) fixative

and paraformaldehyde (PF) fixative. The sampling and microscopy protocols were revised to minimise damage to the inflorescence samples during extraction, transport and storage.

For characterisation of differences in reproductive development in normal and mantled phenotypes, it was proposed comparable samples be collected from ramets of the same clone, in the same environment, on the same day. This was to avoid any environmental biases in the analysis. In order to collect comparable stages, easy identification of the developmental stage is necessary.

Lack of dependable references for field identification of developmental stages makes the sampling of comparable phases difficult. The stage of inflorescence at a particular frond number depends on the frequency of frond emergence, which is affected by genetic and environmental factors. Hence F18 of two palms even of the same clone are not necessarily at the same developmental stage. Hence, it is not recommended to go only by the frond number, but also consider other visual cues for the selection of comparable samples. In addition, the efficacy of length of inflorescence as a field reference of the developmental stage was also examined.

5.2. MATERIALS & METHODS

5.2.1. Plant Material

For optimisation of field sampling and preparation of the reference series, a single normal *Tenera* ramet in the nursery was sampled. The sampling range used for reference series preparation was F9 to F18.

Optimisation of the microscopy protocol was done using representative samples from the non-destructive sampling range (Table 5.1). Four normal palms at the AAR nursery were sampled to obtain female inflorescences from F9, F10, F13 and F17. The stages were specifically chosen so as to trial the full range of processing methods required for the non-destructive sampling range. All conditions and procedures for field sampling, fixation, resin infiltration, embedding, sectioning and preparation of slides were tried.

The protocol formulated via trials was followed for non-destructive (F7 to F18) and destructive sampling (F0 to F20) of selected mature and young palms, with established genotypic and phenotypic identity (Chapter 3). The sampling ranges were influenced by external factors. The samples thus obtained were used for establishing a developmental classification (chapter 6) and construction of a developmental series (Chapter 7).

Non-destructive sampling (F7 to F18) of mature palms was conducted in 8 palms belonging to 2 clones and destructive sampling (F0 to F21) of young palms in 9 palms from 2 clones. To reduce the environmental effect, palms were sampled in groups. Mantled palms were always sampled alongside a normal palm from the same clone within the same replicate. Details of the palms sampled, and the sampling range is included in table 5.1. The

individual sampling ranges were influenced by external factors (such as rotting of inflorescence and practical difficulties related to extraction).

Table 5.1 Sampling Ranges Used. Details of sampling ranges used for optimisation of protocols (1 and 2), the establishment of developmental stages (1-4) and the construction of the developmental series (3 and 4). Clones and palms included in each sampling group is specified along with the phenotypes (N=normal, M=mantled) of the palms.

Phase of Sampling		Details		Sampling Range
1	Preparation of Reference Series	Samples were collected on ice from a single normal *Tenera* ramet. Measurements and fine sectioning were carried out under lab conditions.		F9 to F18
2	Optimisation of microscopy protocol	Samples were collected from four normal palms at the AAR nursery, in two types of fixatives for the microscopy protocol optimisation.		F9, F10, F13, F17
3	Non-destructive Sampling	Group 1	Clone: A229 Palms: 71N, 69M	F8 to F18*
		Group 2	Clone: R291 Palms:18N, 17M	F7 to F18*
		Group 3	Clone: R291 Palms: 15N, 19M	F7 to F18*
		Group 4	Clone: R291 Palms: 16N, 23M	F9 to F14*
4	Destructive Sampling	Group 1	Clone: A478 Palms: 7838N, 7842M	F-29 to F20*
		Group 2	Clone: A478 Palms: 7841N, 7839M	F-29 to F20*
		Group 3	Clone: A366 Palms: 7553N, 7551M,	F-27 to F17*
		Group 4	Clone: A366 Palms: 7536N, 7554M, 7558M	F-27 to F18*

* The sampling ranges were influenced by external factors.

5.2.2. Oil Palm Inflorescence Sampling

Before sampling, Frond 1 was identified, and the arrangement of fronds was established (Chapter 3, section 3.2.2). In the case of selected ramets of established genotypic and phenotypic identity, censuses were conducted the week before sampling to check the sex of the inflorescences. Census could be achieved from F20 to F14.

Non-Destructive Sampling: The ripe and unripe bunches are removed with the harvesting tool. Sampling was started at inflorescence at anthesis

or the closest stage available (roughly F18). Experienced staff climbed to the canopy of the palms and extracted whole inflorescences along with the spathes from the base of numbered fronds (Figure 5.2) under the supervision of the researcher. Once the base of the inflorescence was cut it was lowered to the ground in a bag, minimising physical damage.

Figure 5.2 Inflorescence Sampling from a Mature Palm in the Field. Photos show experienced staff extracting whole inflorescences from a mature palm canopy using cutting tools.

Destructive Sampling: Sampling of older stages was carried out the same way as in the case of non-destructive sampling. The younger fronds were then labelled with frond numbers and tied together. The trunk of the palm was then cut at the base and taken to the lab (Figure 5.3). Younger inflorescences were extracted from the fronds, in the lab (Figure 5.1).

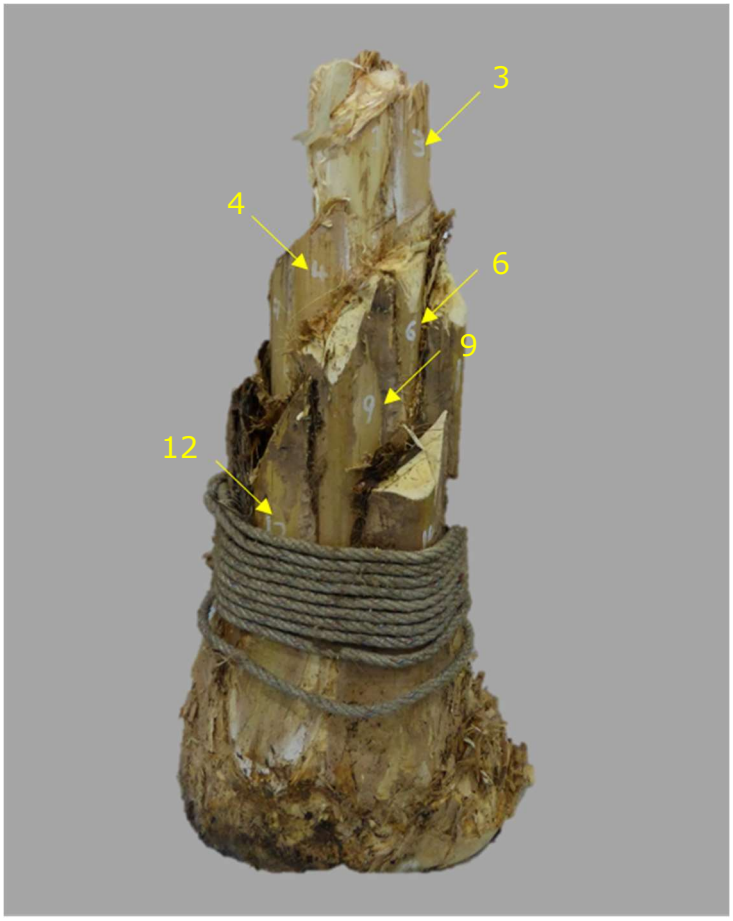

Figure 5.3 Trunk of a Young Palm Cut at the Base with Fronds Labelled. Labelling of fronds as per the naming conventions previously described in chapter 3 is shown by yellow arrows.

5.2.3. Field Sampling Data

Clonal origin, palm identification, phenotype (normal/mantled), frond number, sex of inflorescence, and the length of inflorescence were recorded in the field itself. Palm ID (In the format Clone-Palm) and frond number were used to label the samples. Samples were photographed and physical attributes as per visual staging criteria (see chapter 6) were recorded during field sampling.

5.2.4. Preparation of Reference Series

It was estimated that individual florets from frond 16-18, and spikelets from frond 9- 16 could be collected in the fixative from the field for microscopic analysis (Adam *et al*., 2005). For F14 to F18, where the inflorescence was

too big (over 30cm in length) for the icebox, spikelets from the top and bottom of the inflorescence were collected. The whole inflorescences were sampled for F9 to F13.

The dimensions and weights of spikelets, florets and individual floral organs were recorded (Appendix 4). Observations at 4x magnification were also made using a stereomicroscope (Leica, UK). This data was used as a guideline for estimating the size of containers and amount of fixative required for sampling for microscopy and the extent of separation (individual florets/organ-specific) possible in the field. The reference series was also used for establishing visual staging criteria and characterising the developmental stages (Chapter 6).

5.2.5. Microscopy Protocol Optimisation

For optimisation of microscopy protocol, fine sectioning of samples was done to bring down the size of the samples to less than 2.5cm (for better fixation), under lab conditions. The resultant samples were spikelets of inflorescences from F9 [and F10 as a duplicate], sections of spikelets containing individual floral triads of F13, and halved florets of F17 (Figure 5.4).

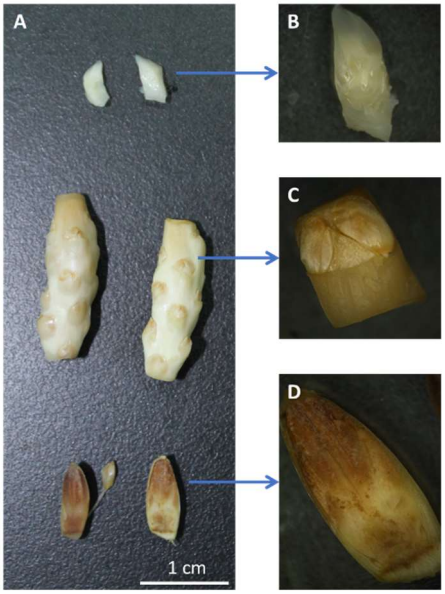

Figure 5.4 Inflorescence Sample Types Used for Microscopic Protocol Optimisation. A. Alongside size comparison of different samples. B. Spikelets from F9 or F10. C. Floral triads cut out from sections of F13 spikelets. D. Halved florets from F17.

5.2.6. Sample Preparation for Histology

In the field, three sectioning methods were practised for the non-destructive sampling range F7 to F18, as described below. The sampling range mentioned is a rough estimation.

a. For F16-F18: separation of florets and sectioning to bring down the size (vertical and horizontal sectioning to 6-8 pieces) for microscopy (Figure 5.5 A).

b. For F12-F15: separation of spikelets and sectioning to obtain floral triads (Figure 5.5 B).

c. For F7-F11: separation of spikelets and sectioning to remove the non-flower bearing top and back for size reduction (Figure 5.5 C).

The fine sectioned samples for microscopy were collected in fixative and was used in the microscopy protocol (Section 5.2.1).

In the destructive Sampling range where removal of spikelets was no longer possible (<F6) the inflorescence was sectioned in four and fixed.

In negative stages (<F0), removal of the peduncular bract and prophyll is not possible without damaging the inflorescence within as the whole structure is minute and soft. Hence the whole inflorescence enveloped in the protective spathes was fixed for microscopy. Sampling for microscopy was possible till F-29 in most cases.

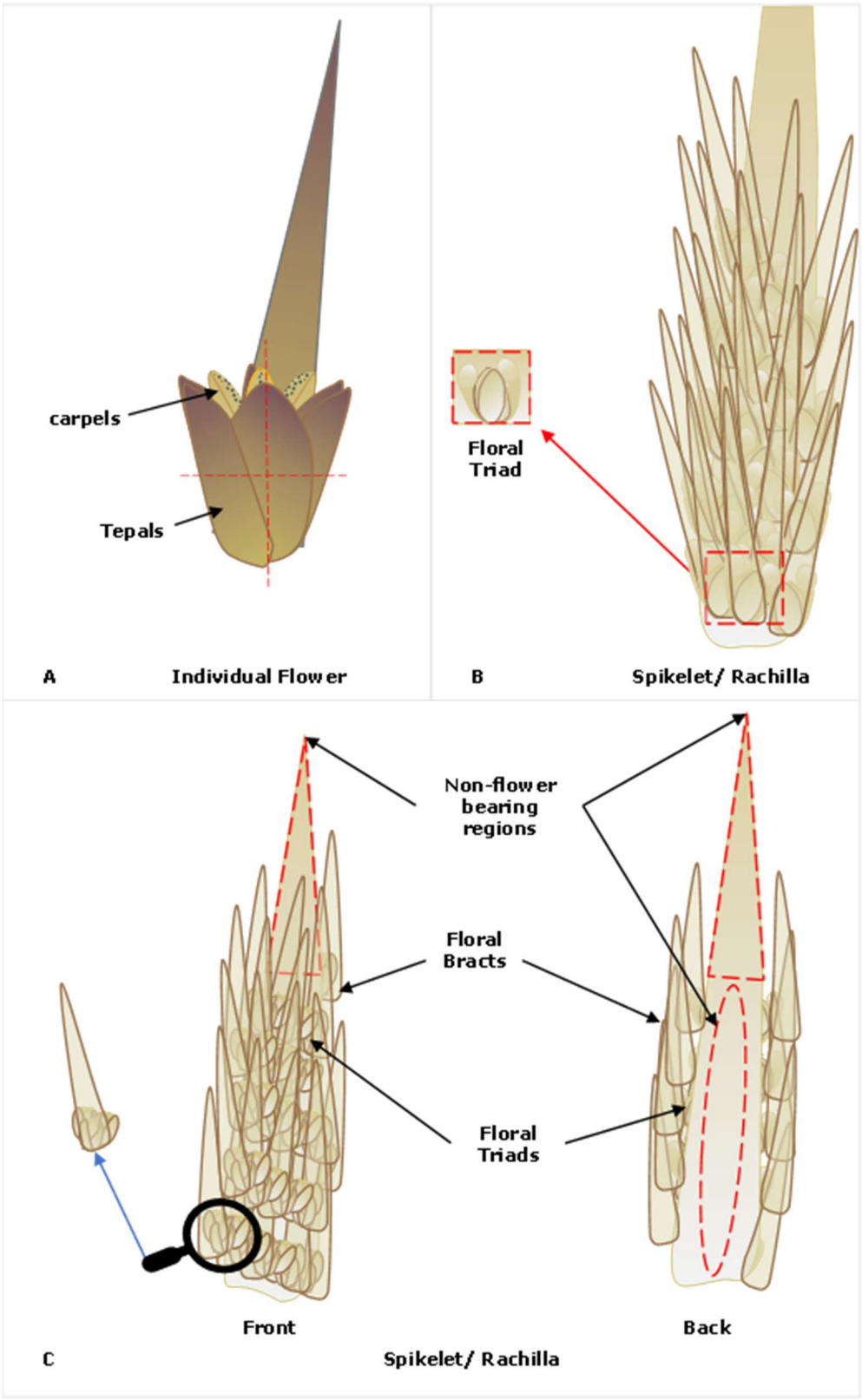

Figure 5.5 Sectioning Methods Practised for the Non-Destructive Sampling Range. (A) sectioning of individual pistilate flowers at F16-F18. (B) Sectioning to extract intact floral triads from the spikelet/ rachilla at F12 to F15. (C) Parts of a spikelet/ rachilla of developing inflorescences showing non flower bearing regions, floral bracts and floral triads (Original Illustration).

5.2.7. Histology

For microscopic examination, the inflorescence samples were sectioned and collected in the fixative at the field. Further fixation and dehydration were conducted at AAR-Biotechnology, Semenyih. Resin infiltration, embedding and sectioning were carried out at the histology facilities of MPOB-biotechnology (No.6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang Selangor). MPOB histology techniques using resin developed by the ORSTOM-CIRAD team LRGAPT (France) were adapted for this. The stained and dried slides were visualised, and images were captured at AAR-Biotechnology. The schematics of the histology protocol is depicted in figure 5.6 and further details are included in the following sections.

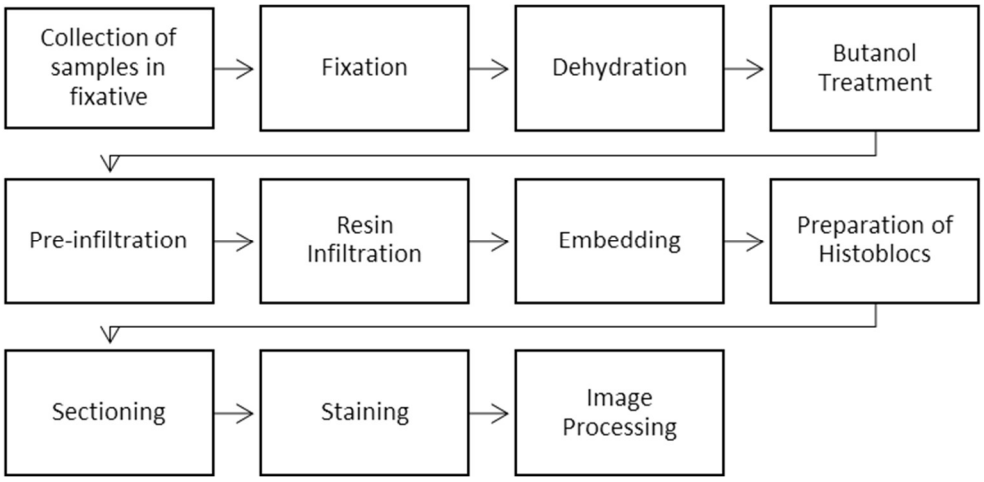

Figure 5.6 Simplified Schematics of Steps Involved in The Histology Protocol.

5.2.7.1. Fixation

The fixatives were prepared fresh every time and kept at 4°C till field sampling. During inflorescence sampling, fine sectioned samples were collected and transported in the fixative. Two fixatives, glutaraldehyde–paraformaldehyde–caffeine (GPC) and 4% paraformaldehyde (PF) were compared for their effectiveness during microscopy protocol optimisation. The latter was used for histology of selected palms.

4% Paraformaldehyde (PF): The 4% Paraformaldehyde fixative was prepared using the CIRAD recipe by dissolving 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) by heating in a water bath for 1.5-2 hours (for recipe see Appendix 5). 0.1% (v/v) each of Triton X-100 and Tween 20 were added while still warm.

Glutaraldehyde-Paraformaldehyde-Caffeine (GPC): The Glutaraldehyde-Paraformaldehyde-Caffeine fixative [10% paraformaldehyde, 25% glutaraldehyde, 0.5g(w/v) caffeine, 0.2M phosphate buffer, pH 7.2] was obtained from MPOB (For recipes for the preparation of the solutions, see Appendix 5).

Samples were fixed for 1 h under vacuum. After which they were moved into fresh fixative and were kept overnight at 4°C on a rotary mixer. The following day the samples were brought to room temperature and washed in two changes 1x PBS for 30 minutes each.

5.2.7.2. Dehydration and Sample Treatment

Fixed samples were dehydrated through a graded ethanol series, then treated with butanol to soften the tissues and pre-infiltrated in a 1:1 mixture of Resin base solution Technovit®7100 and Butanol (Table 5.2).

Table 5.2 Pre-treatments for Histology Samples. The different treatments involved in the dehydration and processing of fixed inflorescence samples prior to resin infiltration are summarised. The percentage of solutions and the duration of treatment are specified.

Treatment			Duration
Ethanol Series	Ethanol	30%	1 hour
		50%	1 hour
		70%	1 hour
		80%	1 hour
		90%	1 hour
		95%	1 hour
		100% 2x	1 hour
Butanol Treatment	Butanol	1st bath	24 hours
		2nd bath	24 hours
		3rd bath	24 hours
Pre-infiltration	Resin base solution Technovit®7100 and Butanol (1:1 mixture)		48 hours

5.2.7.3. Resin Infiltration, Embedding and Preparation of Histoblocs

All solutions were prepared according to the manufacturer's instructions (Technovit®7100, Heraeus Kulzer, Germany). The methodology is illustrated in figure 5.7.

The resin infiltration solution was prepared by dissolving 1g of Technovit®7100 Hardener 1 in 100ml of Technovit®7100 Basic solution (Heraeus Kulzer, Germany). Tissue samples were kept submerged in the impregnation solution for a week at 4°C (Figure 5.7 A).

The polymerisation solution was prepared by mixing 15ml of impregnation solution and 1ml of Technovit®7100 Hardener 2. Embedding moulds were half-filled with the polymerisation solution and the prepared samples were oriented in them. The moulds were then filled fully with the polymerisation solution. The polymerisation was carried out at room temperature overnight (Figure 5.7 B).

The polymerised blocks with embedded samples were glued to HistoBloc carriers, that can be clamped to the microtome's holder, using HistoResin mounting medium (Leica-Reichert Jund 702218-501 and 70-2218-502). The powder and liquid components of the mounting medium were combined to form a viscous mixture. This was used to install the HistoBlocs to the embedding moulds and the Histoblocs were dried for one day. The polymerised blocks now attached to the Histoblocs could be removed from the moulds and were ready for sectioning (Figure 5.7 C).

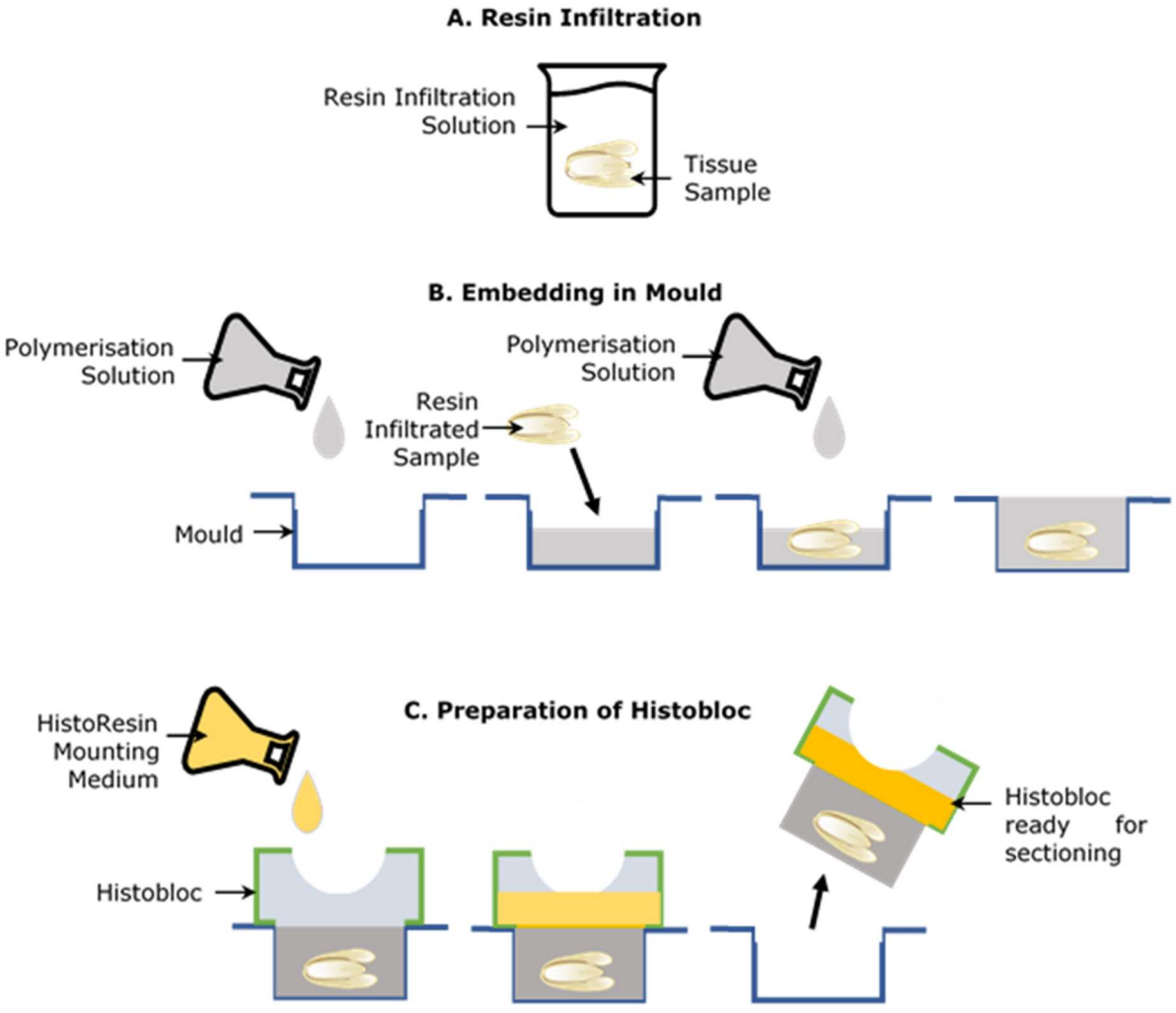

Figure 5.7 Methodology Employed for Resin Infiltration, Embedding and Preparation of Histobloc (Original illustration).

5.2.7.4. Sectioning and Staining

Sectioning was done at 7µm thickness using Leica Rotary Microtome (Leica RM2165, Germany). The resin sections were placed in distilled water taken in a glass container with black paper underneath and were picked up using microscope slides (Sail brand, Cat No. 7105). The slides were dried on an electrothermal slide drying bench. The following day, staining was conducted according to Fisher (1968) with minor modifications (Table 5.3).

Table 5.3 Staining regime. The staining regime used for oil palm inflorescence samples in detailed. The different stains used and the duration of each treatment are specified. Slides were rinsed using distilled water between different stain treatments.

Treatment	Duration
Periodic acid	5 mins
Rinse well with distilled water (pH 4.5)	
Schiff's reaction	20 mins
Rinse well with distilled water (pH 4.5)	
Naphthol blue-black at 60°C	5 mins
Rinse 4x in distilled water	

Periodic acid-Schiff stains polysaccharides red while Naphthol blue-black specifically stains soluble or reserve proteins blue-black (Fisher, 1968. For recipes of stains see Appendix 5). The slides were dried on the wooden stand. Once dry, they were mounted with Surgipath mounting medium (Leica, Germany) inside the hood and cover slides were placed. They were dried overnight before examination. The excess mounting medium was removed using a scalpel.

5.2.8. Image Processing

Images of the stained sections were then viewed and photographed with a ToupCam Camera (xCAM 1080 P-HDMI) attached to an inverted biological microscope (NIB 100). For lower magnifications, a Nikon Digital sight DS-Fi2 camera was used with RaxVision stereo microscope. Inbuilt software was used for image processing.

5.2.9. Statistical Analysis

All statistical analysis of data was carried out using Genstat 64-bit Release 18.2 (PC/Windows 8) Copyright 2016, VSN International Ltd. Model checking was done by plotting the residuals against the standardised residuals for normal distribution. Analysis of variance was performed using generalised linear model.

5.3. RESULTS AND DISCUSSION

5.3.1. Analysis of Field Sampling Data

Inflorescence sampling of selected palms was done in groups and only when normal and mantled palms of the same clone were available within the same replicate under the same environmental conditions. Infected palms and revertants found in mature clones over 10 years of age were excluded.

Inflorescence abortion or rotting is thought to be caused by environmental stress at younger stages and lack of pollination at later stages. It is impossible to foresee abortion/rotting at stages of development where the inflorescence is still enclosed within the base of the fronds. As a result, sampling was not possible for palms belonging to clone R295, and for stages F18 to F16 of palm A366-7554. Further, the regular occurrence of male inflorescences in the normal palms limited the number of comparable stages with its mantled counterpart(s) of the sampling group in which male inflorescences were uncommon (Chapter 9).

Field sampling data (Appendix 6) was analysed to identify field references of the developmental stage. The leaf stage is useful in collecting developing inflorescences in the order of their age. Hence is useful for comparing samples extracted from the same palm. However, the developmental stage of the inflorescences extracted at a specific leaf stage is dependent on the frequency of frond emergence and environmental factors. For instance, in younger palms frond emergence is quicker.

Length of the inflorescence was taken as an indicator of inflorescence growth. The effect of the different factors were analysed by accumulated

analysis of variance (ANOVA), that is: the age of palm (mature or young), clonal origin, phenotype (normal or mantled), leaf stage (frond number) and sex (female, male or hermaphrodite) of inflorescence (Table 5.4).

The length of the inflorescences increased significantly from one leaf stage to the next in all palms as expected. Parallel to the observation made regarding fruit bunches there was a significant difference in inflorescence length between young and mature palms ($p<0.001$). The younger palms tend to produce smaller inflorescences compared to mature palms, the difference in length becoming more pronounced at later stages (Figure 5.8).

Table 5.4 Accumulated Analysis of Variance of Field Sampling Data. Significant effects are denoted with *. Only significant interactions are shown. Frond No. is the leaf stage from which inflorescence was harvested. The age category refers to the age group (mature or young). Phenotypes compared were mantled or normal. Sex was female, male or hermaphrodite.

Source of variation	Degrees of Freedom	Mean Sum of Squares	F value	F probability
Frond No	1	24110.60	673.01	<.001**
Age Category	1	4779.09	133.40	<.001**
Clone	2	104.60	2.92	0.056
Phenotype	1	0.70	0.02	0.889
Sex	2	106.90	2.98	0.053
Frond No*Age Category	1	7897.41	220.44	<.001**
Frond No*Clone	2	303.26	8.47	<.001**
Frond No*Sex	2	377.85	10.55	<.001**
Clone*Sex	2	298.88	8.34	<.001**
Frond No*Clone*Phenotype	2	138.76	3.87	0.022*
Residual	190	35.82		
Total	219	213.28		

At higher leaf stages (that is in the case of older inflorescences) clonal origin had a significant effect on the length of inflorescence($p<0.001$). For example, A229 had longer inflorescences than R291 at anthesis.

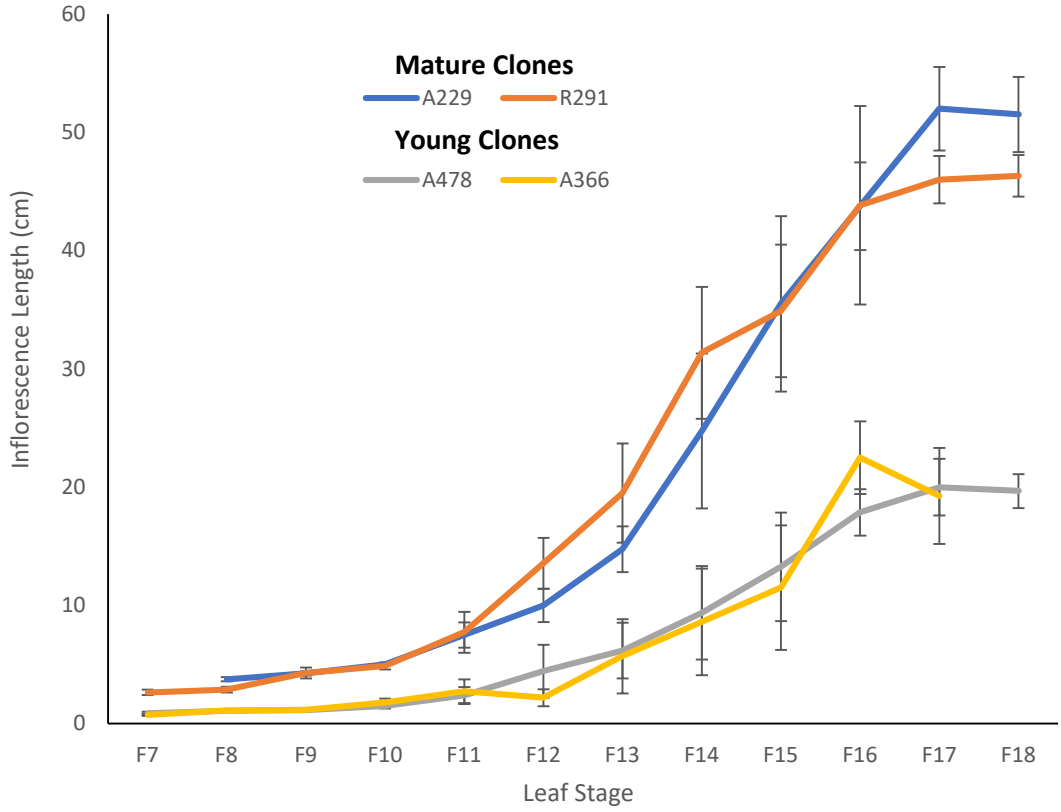

Figure 5.8 Average Length of Inflorescence at Different Leaf Stages. Data shown is for palms belonging to mature clones A229 and R291 and young clones A366 and A478. Error bars show standard deviation associated with the mean values.

Within the same clone, inflorescence length changed significantly with the sex of inflorescence ($p<0.001$) at specific leaf stages (Figure 5.9). At lower leaf stages, the inflorescences are similar in length irrespective of their sex but at higher leaf stages hermaphrodite and male inflorescences were longer compared to female inflorescences at the same leaf stage. Male inflorescences have a longer peduncle, but length values presented here are independent of the length of the peduncle, as the measurements were taken from the start of spikelets and not the point of attachment. This was interesting since Adam *et al*. (2005) had previously reported male and female inflorescences at the same leaf stage to be similar in size, which was not the case in this sample set. Comparison between clones was not possible due to the small number of male/hermaphrodite inflorescence found.

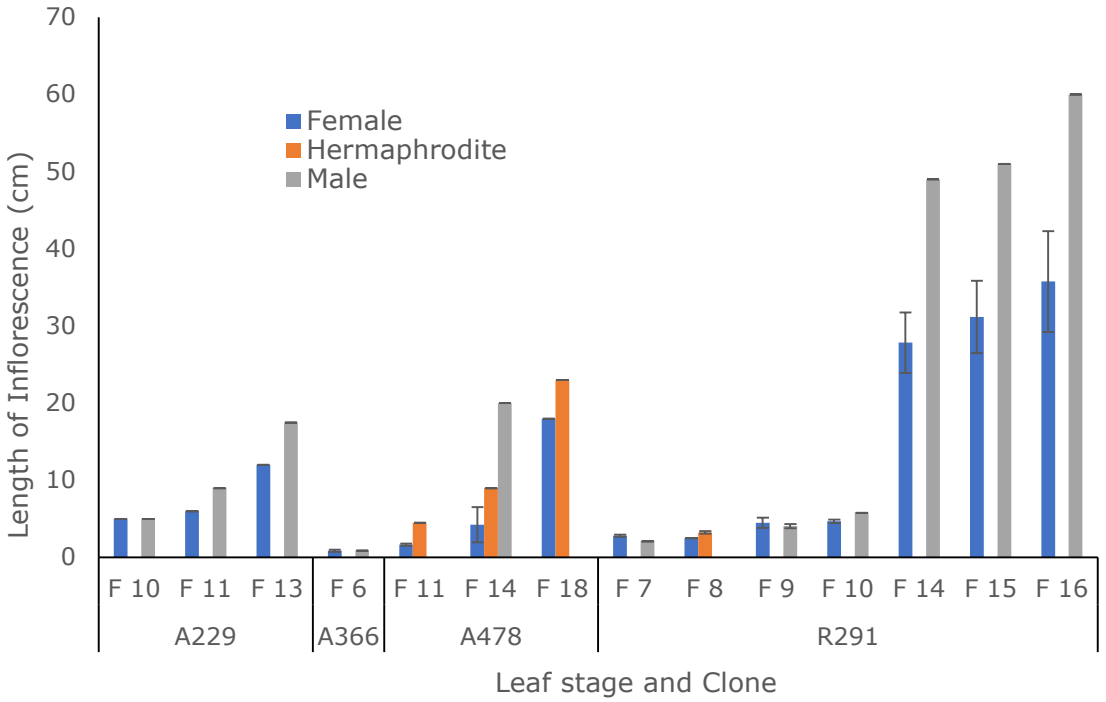

Figure 5.9 Effect of Sex on Inflorescence Length. The average length of inflorescences of different sexes (Female/ hermaphrodite/ male) is shown for different leaf stages where comparison is possible within the clone. Error bars show standard deviation of mean data.

The effect of phenotype (mantled/normal) was significant within clones at certain leaf stages (Figure 5.10). Mantled and normal inflorescences had similar lengths at younger leaf stages but showed notable differences at older leaf stages (F14 and above). In clone A229, normal inflorescences were consistently longer than their mantled counterparts from leaf stages 11 to 16 but shorter at F16 to 18. In clone R291, the normal inflorescences were longer than mantled from F12 to F19. Interestingly in the younger clones A366 and A478, mantled inflorescences were longer than normal inflorescences at similar leaf stages but with high variability between palms.

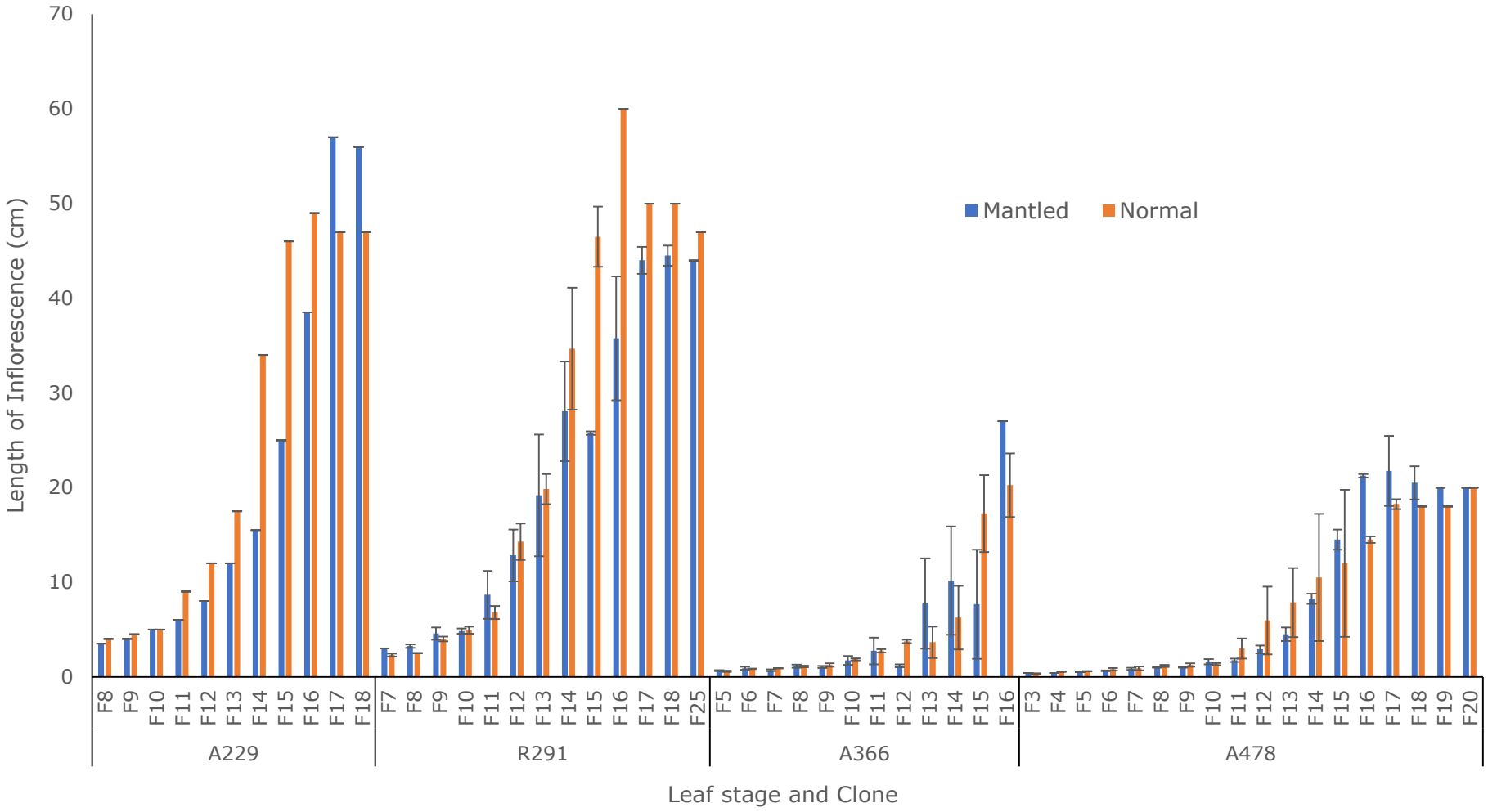

Figure 5.10 Effect of Mantled Phenotype on Inflorescence Length at different Leaf Stages. The average length of inflorescences of different phenotypes (mantled/normal) is shown for different leaf stages where comparison is possible within the clone. Error bars show standard deviation of mean data.

The length of the inflorescence is a good field reference, to ascertain the chronological order of the samples collected from a palm. However, from the analysis, it is evident that the clonal origin, age of palm (mature or young), phenotype (normal or mantled), and sex (female, male or hermaphrodite) affect the length of the inflorescence. Hence length alone cannot be used to ascertain the developmental stage for between palm comparisons. Hence, the samples were further characterised by visual scoring and analysed by histology to develop a developmental classification (Chapter 6).

5.3.2. Effect of Fixatives on Microscopy Protocol

In general, the field sampling and sample processing protocols used minimised damage to the inflorescence samples collected. The different fine sectioning methods could effectively reduce sample sizes for successful fixation. During microscopy protocol optimisation the inflorescence samples were collected in both types of fixatives, glutaraldehyde–paraformaldehyde–caffeine (GPC) fixative and 4% paraformaldehyde (PF) for comparison purposes. Dehydration, embedding with Technovit resin and sectioning were carried out using an identical protocol.

The difference in fixative (PF or GPC) type did not cause a significant difference in the quality of slides produced. However, they were distinguishable with respect to the colour change of tissues. F9 and F10 samples showed distinct yellowing after 24 hours when fixed in GPC (Figure 5.11A). The yellowing is possibly associated with the penetration of glutaraldehyde into the tissue. Further, the browning of tissue at 24 hours and 48 hours after sampling was more prominent in those fixed by PF

compared to those in GPC. The discolouration was most prominent in F17 samples (Figure 5.11 B). It was found that the discolouration could be reduced by collecting samples directly in the fixative in the field. The protocol was adjusted accordingly.

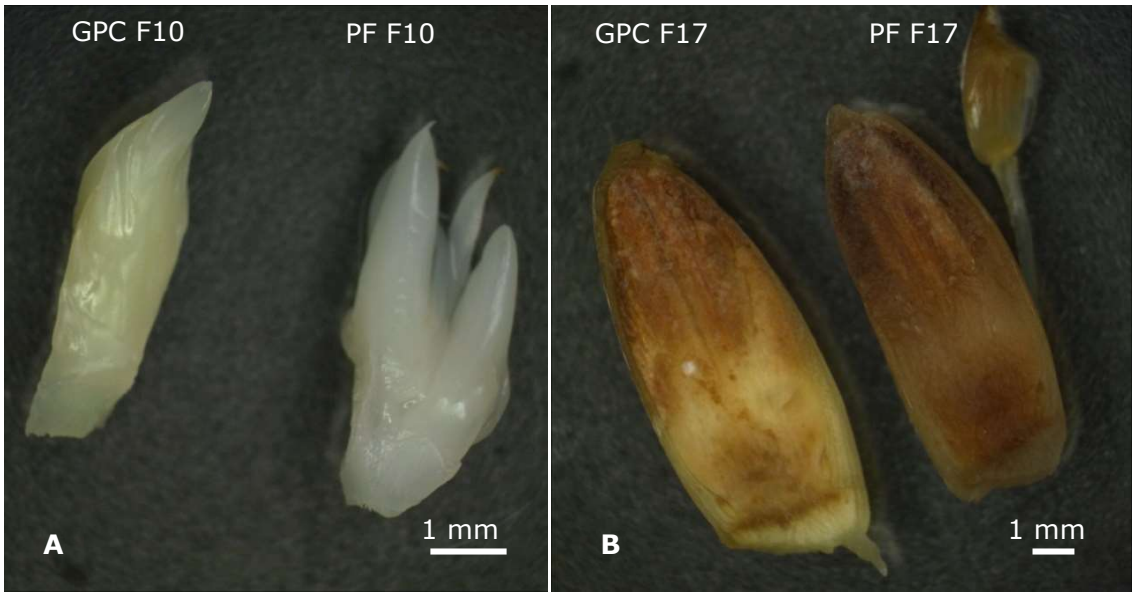

Figure 5.11 Discolouration in Fixed Inflorescence Samples. Discolouration of tissue observed in samples fixed in GPC (left) and PF (right) are shown. (A) shows yellowing of tissue in GPC fixed spikelets from F10; (B) shows increased browning in halved florets of F17 fixed by PF. Labels show fixative used and developmental stage. GPC- Glutaraldehyde Paraformaldehyde Caffeine, PF- 4%Paraformaldehyde.

The tissue was found too hard for microtome sectioning in all samples even after 48 hours of butanol treatment. This caused it to break off from the resin during sectioning, especially in the case of older stages and larger samples (Figure 5.12). Hence neither of the fixative buffers with Technovit resin is recommended for floral developmental stage 3 and above.

FAA 70% with Paraplast X-tra resin as used by de Farias *et al*. (2018) in American oil palm may be a better alternative. de Farias *et al*. (2018) used two fixatives. One, Paraformaldehyde 4% in sodium cacodylate 0.1M at pH 7.0 and second FAA 70% which is a mixture of Formaldehyde 40%(5ml), Ethanol 70%(90ml) and glacial acetic acid (5ml). Technovit 7100 (LKB Pharmacia, Uppsala, Sweden) was the resin used with the former and

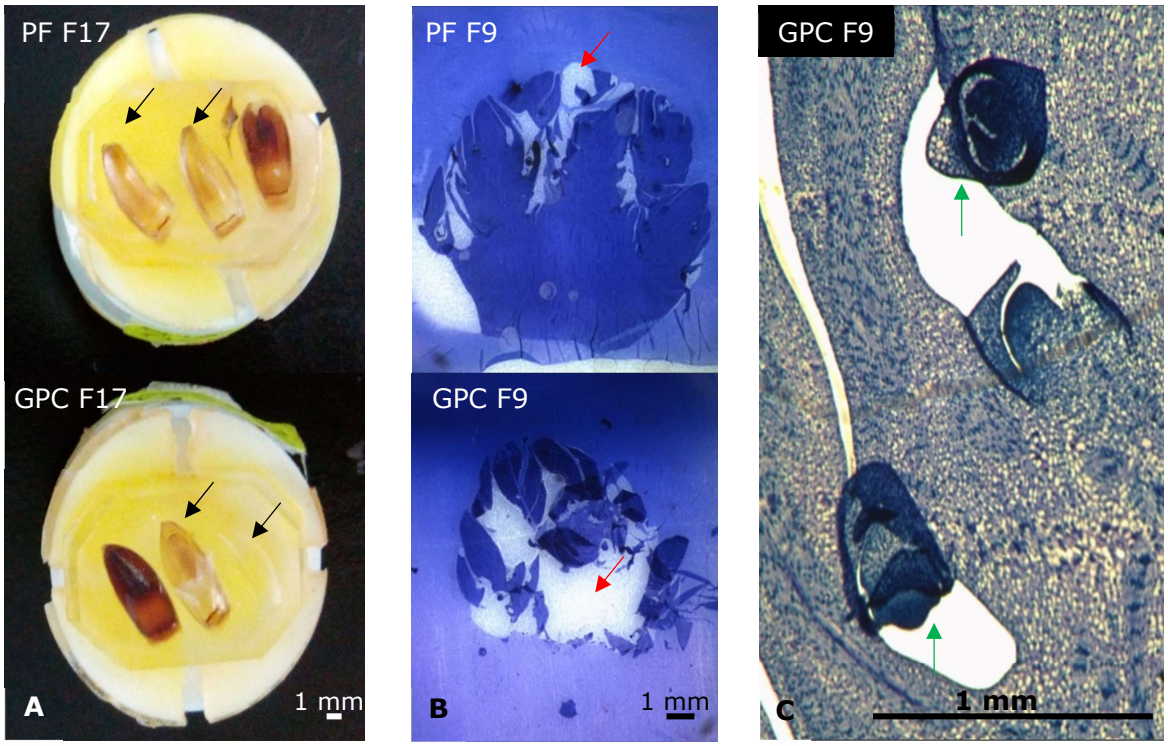

Figure 5.12 Breaking of Embedded Tissue Samples from the Resin during Sectioning. (A) shows embedded floret segments from F17, where the floret segments have dislodged from the mould (black arrows). (B) shows inflorescence sections of F9 as seen through a stereo microscope, where the tissue has detached from the resin (red arrows). (C) shows microscopic images of F9 spikelets where the individual florets have separated from the resin and have folded over (green arrows). Labels show fixative used and developmental stage. GPC- Glutaraldehyde Paraformaldehyde Caffeine, PF- 4%Paraformaldehyde.

Paraplast X-Tra was used with the latter. They reported better penetration of resin with Paraplast. Hence for larger flower samples, Paraplast may be a better alternative to Technovit which fails to infiltrate inner layers of flower tissue at older stages of the inflorescence causing breaking of samples during sectioning.

Nevertheless, the histology method described here proved practical for the histological study of early inflorescence and flower development in oil palm. The staining regime used was especially useful in the detection of meristematic tissue by nuclei/cytoplasm ratio (naphthol blue-black), and starch and polyphenol accumulation in cells (periodic acid- Schiff's reaction).

5.4. SUMMARY

In oil palm inflorescences develop deep within the crown protected by perianths and the base of the subtending frond over a period of 2 to 3 years (from floral initiation to maturity). So, the extraction of inflorescence stages is labour intensive and time-consuming. Optimisation of field sampling protocol helped streamline the process for both non-destructive and destructive sampling ranges and ensured consistency. The unstable nature of mantled phenotype (reversion of mantled palms), occurrence of male flowers in the normal palms (but not in the mantled counterpart), and rotting of inflorescence samples still limited the sampling range and availability of comparable normal-mantled pairs.

Initial characterisation of inflorescence samples was done with field sampling data. Mature and young clones were found to differ in inflorescence length from early on in development. However, within an age group, clonal origin had a limited effect on the length of the inflorescence. Male and hermaphrodite inflorescences were longer than female inflorescences of the same leaf stage. Inflorescence from mantled palms had similar "macro features", that is length and width of the inflorescence and developmental cues, to normal palms. But the data was erratic, possibly due to differential response to environmental cues.

Even though the length of inflorescence can be used to check the accuracy of the numbering of fronds while sampling, on its own it was found not to be a dependable reference for between palm comparisons. The clonal origin, age of palm (mature or young), phenotype (normal or mantled), and sex (female, male or hermaphrodite) affect the length of the inflorescence, in

addition to various environmental cues. Hence length alone cannot be used to find comparable samples between clones or even between palms. Hence a detailed developmental classification of samples, as discussed in chapter 6, is required to enable comparisons between palms and clones.

The difference in fixative (PF or GPC) type did not cause a significant difference in the quality of slides produced. However, the microscopy optimisation trial was helpful in planning more effective sampling procedures. Consequently, the fine sectioning method was carried out in the field itself as this minimised damage to the floral tissue. The samples were collected and transported in the fixative thus reducing discolouration of the tissue. The histology protocol was suitable across developmental stages, except for mature flowers/ fruit stages due to the toughness of the tissue.

CHAPTER 6
DEVELOPMENTAL CLASSIFICATION OF OIL PALM INFLORESCENCES

6.1. INTRODUCTION

Objectives:

- Developmental classification of collected samples to identify comparable normal and mantled samples from each sampling group and detailed characterisation of oil palm inflorescence developmental stages for easy prediction based on field observations and histology.

Oil palm inflorescences develop within the base of developing fronds. Each successively produced frond carries a female, male or hermaphrodite inflorescence. Development of new fronds as well as the inflorescence within takes 2-3 years and nearly two fronds (and inflorescences) emerge from the crown each month. So, in a palm many inflorescences develop in parallel but at different phenological or developmental stages. To compare the reproductive development of normal and mantled phenotypes, normal and mantled palms were sampled in parallel and inflorescence samples were obtained at a range of developmental stages. However further characterisation was necessary to identify comparable samples at equivalent stages of development.

The lack of dependable references for field identification of developmental stages made the sampling of comparable phases difficult. Frond number only gives an indication of the order of emergence of the inflorescences and not the developmental status. The frequency of frond emergence is

influenced by the clonal origin and age of the palm. New fronds emerge from the spear more frequently in younger palms. On the other hand, dry conditions reduce the rate of expansion of fronds (Williams, 1975). Hence, the developmental stage of inflorescence at a specific frond number is determined by genetic and environmental factors. So, sampling the same frond number of two palms did not always yield inflorescences at the same developmental stage.

Length of inflorescence gives a clear indication of inflorescence development and expansion. However, the selection of samples with the same length from different palms also did not yield equivalent samples. The length of the inflorescence was found to be influenced by multiple variables (more details in chapter 5). Hence in the current sample set both frond number and length of inflorescence were found to be unsuitable field references on their own, for the selection of equivalent inflorescence samples.

An inflorescence stage predictive algorithm was formulated by Sarpan *et al*. (2015) based on previous studies on reproductive development in oil palm by Adam *et al*. (2005). The algorithm considered the age of the palm and the length of the inflorescence for categorisation. The algorithm was useful in the classification of the collected samples into five developmental intervals. But these intervals were found to be too broad for early stages of inflorescence development as well as those closer to maturity. Hence, it was proposed other visual cues should also be taken in conjunction for the selection of comparable samples. A robust visual staging based on visible attributes of the inflorescence was used for further classification into eight categories for the non-destructive sampling range. An additional four

categories for early inflorescence development accessible by destructive sampling was established based on histology. Length of inflorescence was used as the primary indicator of growth and was examined across the developmental categories.

To ensure the differences observed were only due to the abnormal phenotype under enquiry, mantled, and not due to clonal (Jaligot *et al*., 2011; Rival, 2018) or environmental differences (Adam *et al*., 2011; Combres *et al*., 2013) sampling was done in groups. Each mantled palm was sampled along with a normal counterpart belonging to the same clone from the same replicate. In the initial analyses, phenotype (Mantled/Normal) had an inconsistent but significant effect on the length of inflorescence, within clones (Chapter 5). This effect of mantled phenotype on length of inflorescence was examined within the prescribed developmental categories.

6.2. MATERIALS & METHODS

6.2.1. Plant Material

Initial assessments were made using the reference series prepared by selective sampling (Chapter 5 and Appendix 4). Further, the data collected from 4 different clones during field sampling was statistically analysed to arrive at the final developmental stage classification. Details of sampling and data collection are included in chapter 5, and Appendix 6. Classification of younger inflorescence stages was achieved via histological analysis (protocol is described in chapter 5, sections 5.2.7 and 5.2.8). Photographs of selected inflorescence samples from each developmental category are included at the end of this chapter in plates 6.1 to 6.10.

6.2.2. Developmental Stage Prediction

The statistical model for the prediction of flower development in oil palm proposed by Sarpan *et al*. (2015) was used on the samples collected. Using the probabilities generated by the prediction model the inflorescences were sorted into specific developmental intervals (Appendix 6).

Event probabilities for stages were calculated as follows:

$$P(Y_i > j) = \frac{\exp(\hat{\beta}_{0i} - 1.058Age + \hat{\beta}_{1j}Length)}{1+\exp(\hat{\beta}_{0j} - 1.058Age + \hat{\beta}_{1j}Length)}, \text{ j=1,2,3,4:}$$

where P is the probability, j is the flower stage category (j=1,2,3,4 and corresponds to key developmental stages described by Adam *et al*., 2007; 2005), $\hat{\beta}_{0j}$ and $\hat{\beta}_{1j}$ are the intercepts (constant) and logit coefficients for length respectively (Table 6.1). A probability values of > 0.5 specifies that

the predicted stage for the input is older than the corresponding j, that is $Y_i > j$ (Sarpan *et al*., 2015).

Table 6.1 Values of $\beta\hat{}_{0j}$ and $\beta\hat{}_{1j}$. The intercepts ($\beta\hat{}_{0j}$) and the logit coefficients for length ($\beta\hat{}_{1j}$) r for flower stage categories (j=1,2,3,4) as prescribed by Sarpan *et al*. (2015) for the calculation of probability values are shown. A probability values of > 0.5 specifies that the predicted stage for the input is older than the corresponding j.

j	$\beta\hat{}_{0j}$	$\beta\hat{}_{1j}$
1	0.838977	2.791197
2	-1.19031	1.450985
3	-4.70567	1.10248
4	-6.32202	0.659079

6.2.3. Visual Staging

Visual Staging was done based on multiple visual traits to describe 8 distinct developmental categories within the non-destructive sampling range (Figure 6.1). Visual criteria used for staging included morphological (pigmentation, size) and physiological (sex, maturity) characteristics revealed through the detailed examination of the inflorescence and its components - the spikelets, the floral triads and the individual floral organs (tepals and carpels). Stages were cross referenced to photographs in the reference series (Appendix 4).

Category 8, Unripe Bunches (Plate 6.1) were used for phenotyping (Chapter 3), Inflorescence samples from categories 1 to 6 (Plates 6.3 - 6.8) were used for microscopic analysis (Chapter 7).

Category 1, Young Inflorescence (Plate 6.8), was further subdivided into 4 developmental stages based on histology: 1A. the development of spathes, 1B. development of spikelet bract primordia, 1C. development of spikelet primordia, and 1D. the development of floret primordia.

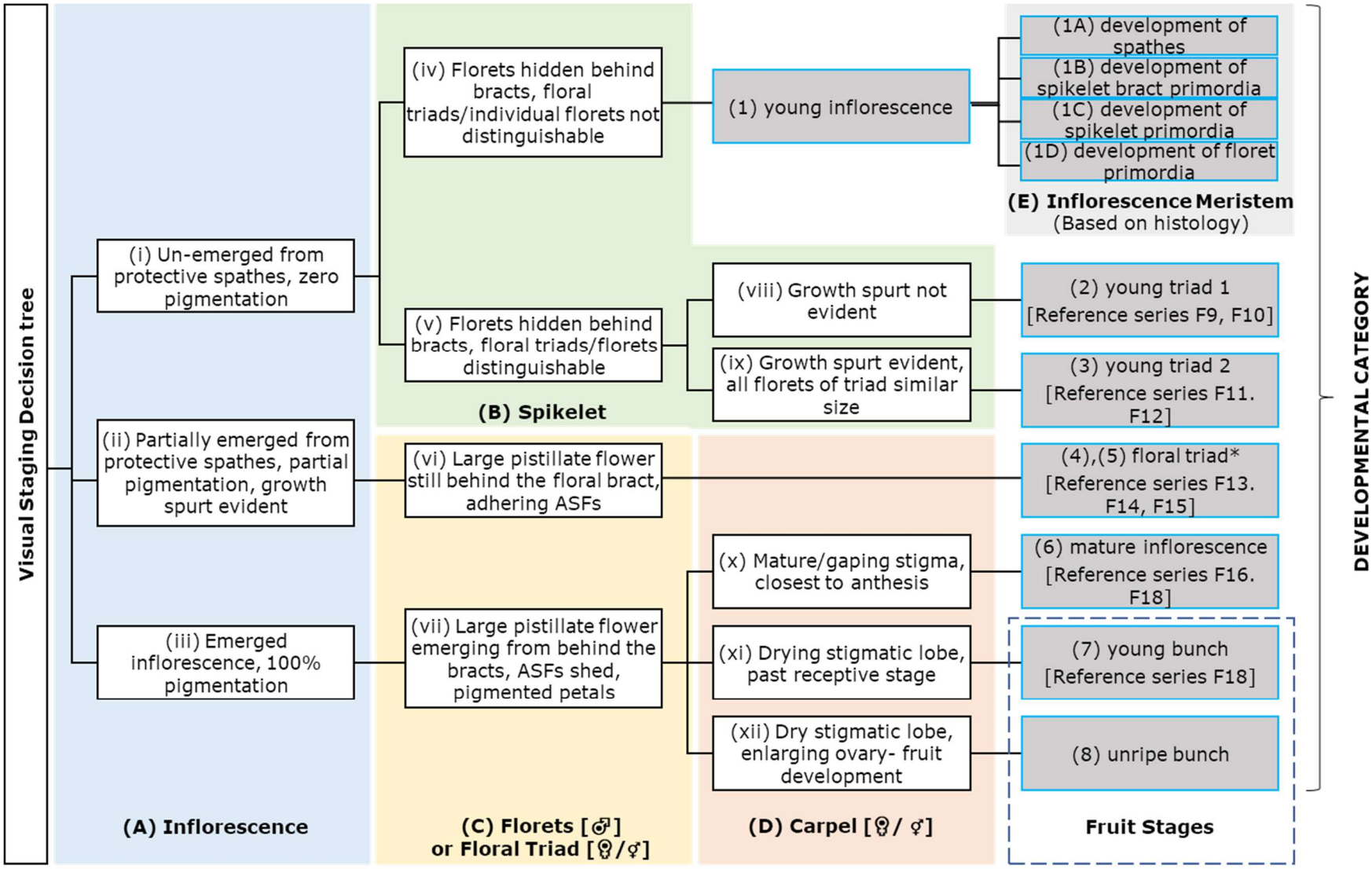

Figure 6.1 Visual Staging Decision Tree. (A)-(E) site of observation (i)-(xii) visual traits used for categorisation, (1)-(8) developmental categories. Developmental Category Floral triads were further divided into two (Category 4 Floral triad 3 and category 5 Floral Triads 4) based on developmental intervals prescribed by the predictive algorithm. ASF- Abortive staminate flowers

6.2.4. Statistical Analysis

All statistical analysis of data was carried out using Genstat 64-bit Release 18.2 (PC/Windows 8) Copyright 2016, VSN International Ltd. Model checking was done by plotting the residuals against the standardised residuals for normal distribution. Regression analysis using generalised linear model was used unless otherwise specified.

6.3. Results and Discussion

6.3.1. Developmental Classification

Using the probabilities generated by the statistical prediction model (Sarpan *et al*., 2015), the inflorescences were sorted into specific developmental intervals (Appendix 6). However, these intervals were found too broad with respect to developmental events. Hence a further categorisation was done based on visual staging (Section 6.2.3).

The predictive algorithm took into account the age of the palm and the length of the inflorescence. Visual staging criteria used a comprehensive strategy to account for the various developmental cues observable in the field. Hence, the developmental classification arrived at based on the predictive algorithm and visual staging was effective not only in classifying samples into comparable groups (Figure 6.2) but also provided a clear indication of the developmental events, as elaborated in the following sections.

The sampling range was classified into eight categories based on both the predictive model and visual staging (Table 6.2). Mature and young palms were analysed separately, considering the significant difference in inflorescence length between them (Figure 6.2). Fisher's protected least significant difference test for mean separation was performed to assess the effectiveness of categorisation for female inflorescences of mature and young clones (Table 6.2, 6.3, 6.4). Developmental stages had an F probability <0.001 in both mature and young palms; however, all eight categories showed a significant difference of means only in young palms.

Table 6.2 Description of Developmental Categories. The developmental categories are defined based on mathematical prediction and visual staging for female and male oil palm inflorescences.

Category	Predicted Interval	Visual Stage	Description	
			Female	Male
1) Young inflorescence <1	<1	YI	Un-emitted inflorescence with 0% pigmentation Before florets emergence from under floral bracts Not possible to distinguish floral triads by the naked eye	Un-emitted inflorescence with 0% pigmentation Before florets emergence from under floral bracts
2) Young triad 1	1 to 2	YT1	Un-emitted inflorescence with 0% pigmentation Spikelets carry floral triads with a middle female flower and two abortive staminate flowers (ASFs) still enclosed by the floral bract All three florets of the triad are approximately the same size	
3) Young triad 2	2 to 3	YT2	Un-emitted inflorescence with 0% pigmentation Spikelets carry floral triads still enclosed by the floral bract All three florets of the triad are approximately the same size At or before the growth spurt	
4) Floral triad 3	3 to 4	FT3	Prophyll torn, growth spurt evident Floral triads with a larger middle female flower and two adhering ASFs emerging from under the floral bract	
5) Floral triad 4	>4	FT4	Prophyll torn, pigmentation and growth spurt evident Floral triads with a larger middle female flower and two adhering ASFs emerging from under the floral bract	Inflorescence emerging from ruptured prophyll and peduncular bract More than 50% pigmentation Staminate flowers with pigmented tepals emerging from behind floral bracts
6) Mature flower >4	>4	MF	Inflorescence emerging from ruptured spathes (prophyll and peduncular bract) More than 50% pigmentation ASFs shed Pistillate flowers with pigmented tepals emerging from behind floral bracts Mature gaping stigma (at anthesis)	
7) Young bunch >4	>4	YB	Fruit stage Spathes no longer enclosing the bunch Almost 100% pigmentation of the bunch Flowers/fruits with pigmented tepals Stigmatic lobe past receptive stage and drying	Drying and necrotic inflorescence
8) Unripe Bunch	>4	UB	Fruit stage Spathes no longer enclosing the bunch Almost 100% pigmentation of the bunch Flowers/fruits with Pigmented tepals Dry necrotic stigmatic lobe Presence of developing kernel	NA

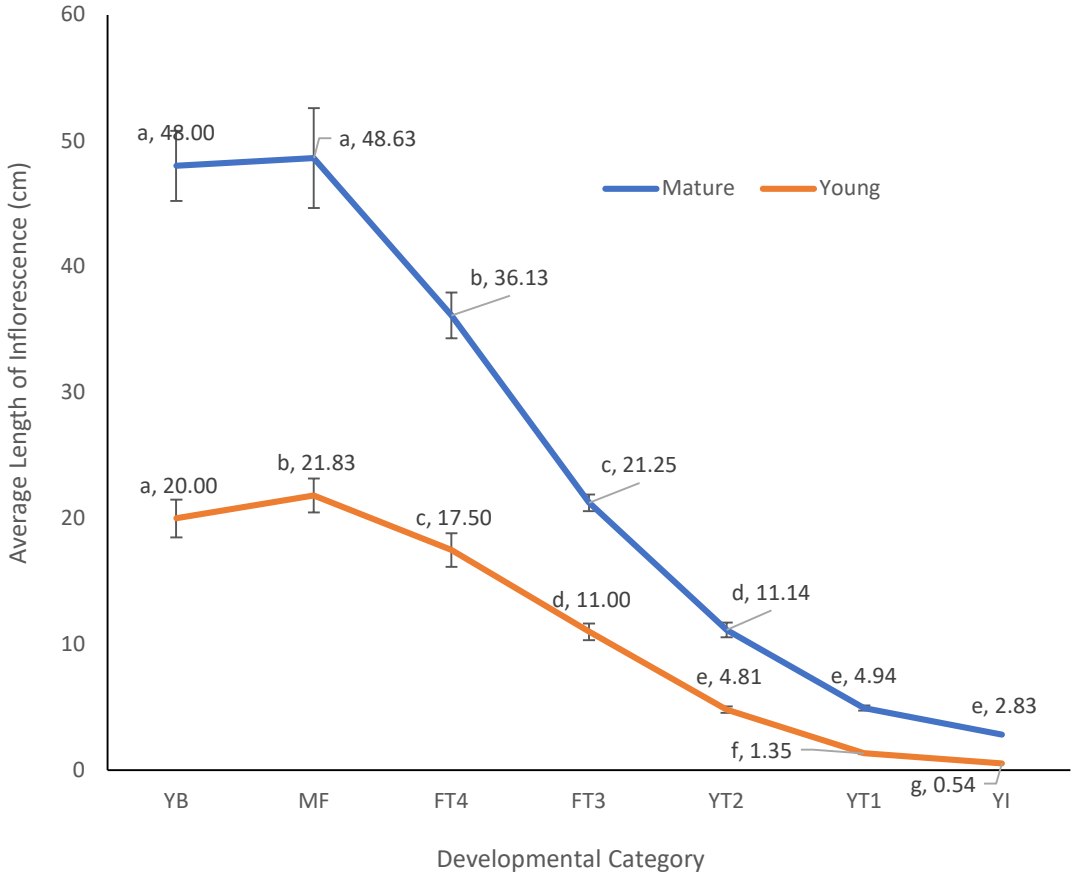

Figure 6.2 Average Inflorescence Length of Developmental Categories. Error bars show standard deviation. Data labels showing means with letters as per Fisher's protected least significant difference test.

Table 6.3 Analysis of Variance of Inflorescences Length of Developmental Categories. Analysis showed a significant difference in inflorescence lengths between the different developmental categories in both mature and young palms.

A. Mature palms

Source of variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F value	F probability
Developmental Category	6	17457.72	2909.62	188.10	<.001
Residual	51	788.91	15.47		
Total	57	18246.62			

B. Young palms

Source of variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F value	F probability
Developmental Category	6	5747.40	957.90	701.57	<.001
Residual	116	158.38	1.37		
Total	122	5905.79			

Table 6.4 Mean Length of Inflorescences within Developmental Categories. The mean length of inflorescence and standard deviation for each developmental category of inflorescences is shown for mature and young palms. Category predictions as per Fisher's protected least significant difference test is included as letters a to e in mature palms and a to g in young palms.

Developmental Category	Mature Palms			Young Palms		
	Mean	Standard Deviation	Category	Mean	Standard Deviation	Category
YB	48.00	1.73	a	20.00	0.00	a
MF	48.63	5.55	a	21.83	2.99	b
FT4	36.13	7.95	b	17.50	2.70	c
FT3	21.25	3.62	c	11.00	2.68	d
YT2	11.14	1.31	d	4.81	1.31	e
YT1	4.94	1.17	e	1.35	0.52	f
YI	2.83	0.41	e	0.54	0.14	g

In mature palms, developmental categories young bunch and mature inflorescence, and young triad 1 and young inflorescence did not show a significant difference of means between categories. But this could be because the developmental categories young bunch and young inflorescence were not well represented in the sample set as they mark the boundaries. Young bunches were post anthesis and can be considered a fruit stage. Microscopy of these stages was difficult since the tissue was big and hard for effective sectioning (Chapter 5, section 5.3.2). Hence it was avoided during sampling. On the other hand, not many inflorescences belonging to the young inflorescence category could be harvested by the non-destructive method of sampling (Chapter 5, section 5.2.2).

The developmental classification was applicable to all the clones, mature and young, used in the present study. It showed good discriminatory power and provided accurate developmental categories (Figure 6.3) within which comparisons were possible within and across clones.

Legend

Clone- Palm Phenotype Frond Number
Developmental Category
Predicted Interval

A229-71N F18	A229-71N F17	A229-71N F16		A229-71N F15	A229-71N F14			A229-71N F12	A229-71N F09	A229-71N F08		
YB	YB	MF		FT2	FT2			YT2	YT1	YT1		
>4	>4	>4		>4	>4			2 to 3	1 to 2	1 to 2		
		A229-69M F18	A229-69M F17		A229-69M F16	A229-69M F15	A229-69M F14	A229-69M F13	A229-69M F12	A229-69M F11	A229-69M F10	A229-69M F09	A229-69M F08
		MF	MF		FT2	FT1	FT1	YT2	YT1	YT1	YT1	YT1	YI
		>4	>4		>4	3 to 4	3 to 4	2 to 3	1 to 2	1 to 2	1 to 2	1 to 2	<1

Figure 6.3 Comparable Samples from Sampling Group 1. The Inflorescence samples collected from normal (71N) and mantled (69M) palms of clone A229 have been classified into 7 developmental categories based on the predictive algorithm and visual staging. Each Cell denotes an inflorescence sample designated by the clone-palm, phenotype(N/M) and frond number(F##). Developmental categories are denoted by initials and colours, YB for Young Bunch, MF for Mature Inflorescence, FT1&2 for Floral Triad 1&2, YT1&2 for Young Triad 1&2 and YI for Young Inflorescence. Predicted intervals based on the predictive algorithm (>4, 3 to 4, 2 to 3, 1 to 2 and <1) are included at the bottom.

6.3.2. Characterisation of Developmental Categories

Category 8, Unripe Bunches (Plate 6.1) were used for phenotyping, a detailed characterisation of them is included in chapter 4. Samples from categories 1 to 6 were used for microscopic analysis, for the preparation of a comparative developmental series between normal and mantled phenotypes. The particulars of these developmental categories are included below.

6.3.2.1. Young Bunch

Samples collected were categorized as Young Bunch (YB) where the predictive algorithm placed them in a developmental interval >4 (or fruit stage 1) and visual staging showed attributes pertaining to the transition from flower to fruit (Table 6.2, Plate 6.2). In these samples, the prophyll and peduncular bract that protects the developing inflorescence had ruptured, and there was more than 50% pigmentation on the bunch, the tepals were dark pink or violet in colour, the abortive staminate flowers had already been shed, the primary female florets had dry stigma lobes and, in some cases, there was an indication of an enlarging ovary (Plate 6.2).

Only a limited number of young bunches (7) were sampled for the project, as they seldom came within the sampling range. However, this category of samples was still found and sampled at leaf stages F17 to F18 in mature clones and F19 and F21 in young clones. At this developmental stage, there was a marked difference in the length of the bunches between the age categories of palms (Mature/Young). Young bunches from mature palms had an average length of 48± 1.73 cm whereas young bunches from young palms were only 20 ± 0 cm long (Table 6.4).

In young bunches from mantled palms, the supernumerary carpels were visible to the naked eye and could be phenotyped. However, microscopy sectioning of samples taken at this stage was too difficult, since the samples were big and too hard even after butanol treatment and an extended period of resin infiltration.

6.3.2.2. Mature Inflorescence

Mature Inflorescences (MF) characterized by florets out of their protective bracts and pigmented tepals were also placed in >4 (or stage 5) developmental interval by the predictive algorithm (Table 6.2, Plate 6.3). The stigmatic lobes were gaping indicative of post anthesis. In mature clones, this stage was seen from F16 to F18 with an average length of the inflorescence 48.63 ± 5.55 cm. In young clones, mature inflorescences were seen from F14 to F19 with a significantly different (F pr <0.001) average length of 21.83 ± 2.99 cm (Table 6.4). Interestingly at this stage different clones showed significant differences (F probability 0.001) in inflorescence lengths and within a clone, there was a noticeable difference in length between mantled and normal palms (Figure 6.2). While mature clone R291 and young clone A366 had longer normal inflorescences, mantled palms of mature clone A229 had longer inflorescences than its normal counterpart. Further examination of this relationship is explored in section 6.3.5.

6.3.2.3. Floral Triads

In a developing pistillate inflorescence, the spikelets carry floral triads with a middle female flower flanked by two abortive staminate flowers (ASFs) (Table 6.2, Plate 6.4, 6.5). At younger stages, the triad was enclosed within the bract. Inflorescences where the ASFs were still present, and the mid

female flower was bigger than the ASFs were categorized as "Floral Triads". They fell in predicted stages 3 or 4. Sampled of stage 4 floral triads (Floral Triad 4 or FT4) showed slight pigmentation.

In mature palms, stage 4 floral triads could be found from F14 to F16. These inflorescences had an average length of 36.13 ± 7.95 cm. Whereas inflorescences categorized as stage 3 floral triads (Floral Triad 3 or FT3) were extracted from F12 to F15 and had an average length of 21.25± 3.62 cm (Table 6.4).

In young palms, stage 4 floral triads were extracted from F13 to F18 and stage 3 floral triads from F14 to F16. They had an average length of 17.5 ± 2.7 cm and 11 ± 2.68cm respectively (Table 6.4). While the length of inflorescence was significantly different between mature and young palms the difference was less pronounced in stage 3 floral triads (F probability <0.001 and 0.003 for stage 4 and stage 3 respectively). The difference in inflorescence length between clones or mantled and normal palms was not significant (Figure 6.4).

6.3.2.4. Young Triads

Samples were categorised as young triads where all three florets of the triad were approximately the same size (Table 6.2, Plate 6.6, 6.7). This meant sex specific developmental changes, be it the development of carpel in the middle female flower or the development and arrest of anthers in the flanking male flowers, had not occurred yet. A sudden exponential hike in the length of the inflorescence was observed at this stage or shortly after. This growth spurt has been associated with organogenesis (Adam *et al*., 2005).

This stage could be further divided into stage 2 or stage 1 based on the predictive algorithm. Stage 2 young triads (Young Triad 2 or YT2) were closer to the growth spurt. They were found at F12 or F13 in mature palms. Their recorded lengths were 11.14 ± 1.31cm. In young palms, the range and length were F11 to F14 and 4.81 ± 1.31 cm, respectively (Table 6.4).

In mature palms, stage 1 young triads (Young Triad 1 or YT1) were found within the range F8 to F12 and had an average length of only 4.94 ± 1.17 cm. In young palms, the range and length were F4 to F15 and 1.35 ± 0.52 cm respectively (Table 6.4). The difference in the length of inflorescences between age categories (mature and young palms) was still significant at young triads stages (Figure 6.3, Table 6.4).

Most samples collected, 70 out of 180, belonged to Stage 1 young triads. Despite the high number of samples, the category also had the second lowest standard deviation for the length of the inflorescences (Table 6.4).

6.3.2.5. Young Inflorescence

The predictive algorithm places all stages of early inflorescence development in the development interval <1, these stages were categorised as young inflorescence (YI). In these floral triads of pistillate flowers could no longer be distinguished by the naked eye. (Table 6.2, Plate 6.8).

Based on histology, they could be further classified as the development of spathes (1A), development of spikelet bract primordia (1B), development of spikelet primordia (1C) and development of floret primordia (1D) (See Chapter 7).

In mature palms, young inflorescences were found at the end of the sampling range at F7 and F8. The average length of the inflorescence was only 2.83± 0.41, the lowest length average and lowest standard deviation in the data set (Table 6.4). In young palms, young inflorescences were collected latest from F10 (upper boundary of the stage) and had a maximum length of 3.5cm. The removal of prophyll and peduncular bract was possible for the upper subset of these samples (F10 to F0). Hence length was measured only for these during sampling. For the lower sampling range (F7), inflorescence samples collected from young palms had an average length of 0.64 ± 0.09 cm. Statistical analysis was not performed due to the skewness of the partial data set.

6.3.3. Developmental Classification of Male Inflorescences

Visual cues associated with developmental changes in male inflorescences were different from their female counterparts hence the same visual staging criteria (Table 6.2) were not effective. However, the predictive algorithm could be used to categorise the samples into five different developmental intervals (Table 6.5, Plate 6.9). Inflorescences of young clones were smaller compared to inflorescences of mature clones at the same frond numbers and developmental stages (Figure 6.1, 6.2). Until developmental stage 4, un-emerged inflorescences with zero pigmentation were observed. In these, the florets were under floral bracts and hence not visible.

Due to the small sample size statistical analysis of the length of male inflorescences was not possible. However, an examination of available data (Figure 6.4, Table 6.5) suggests development similar to the female inflorescences. Within the non-destructive sampling range, a gradual

increase in length at initial stages followed by an exponential increase in length from developmental stage 3 was observed. The exponential increase in the length of the inflorescence was associated with organogenesis, followed by the rapid development of the individual flowers.

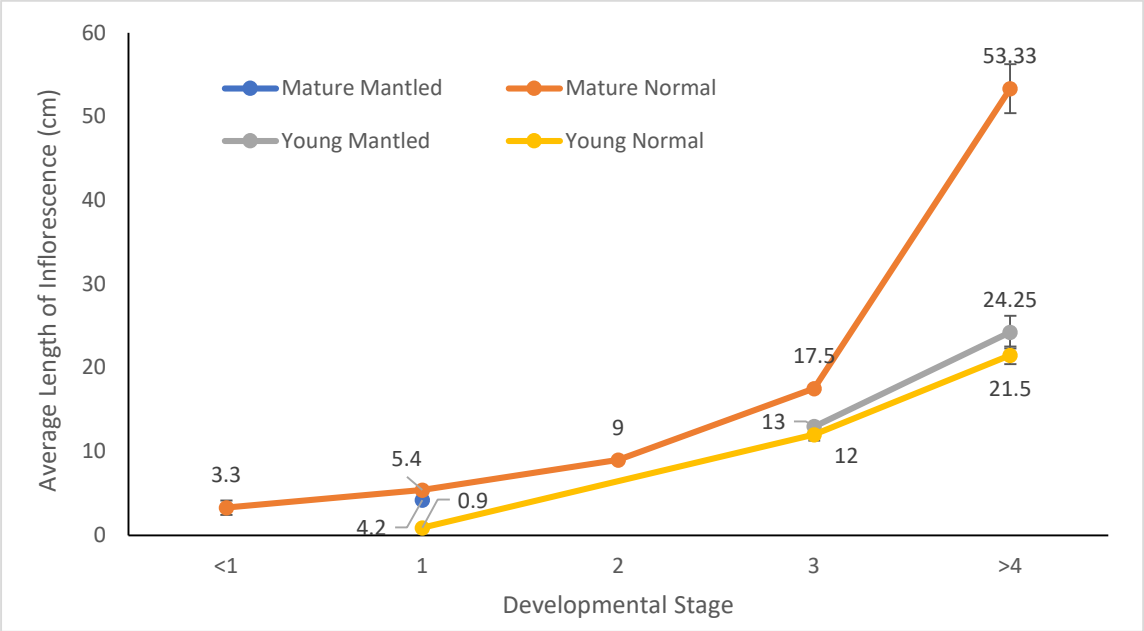

Figure 6.4 Length of Male Inflorescences at Different Developmental Stages. The developmental stages are set based on the intervals predicted by the predictive algorithm. Data shown are the averages of all male inflorescences obtained from normal and mantled palms belonging to mature and young clones. Error bars, where shown, represent the standard deviation associated with the mean values.

Table 6.5 Details of Male Inflorescences at Different Developmental Intervals. The developmental intervals are derived using the predictive algorithm. The number of male samples obtained and the range of leaf stages where male samples were found are specified in both mature and young clones. The average length of inflorescences are expressed as mean ± standard deviation

Developmental Interval	Number of Samples	Range of leaf stages			Average Length (cm)
Mature Clones	11	7	to	16	
<1	3	7	to	15	3.30 ± 1.70
1 to 2	3	9	to	10	5.00 ± 0.80
2 to 3	1	11	to	11	9.00 ± NA
3 to 4	1	13	to	13	17.50 ± NA
>4	3	14	to	16	53.33 ± 5.86
Young Clone	8	6	to	17	
1 to 2	1	6	to	6	0.90 ± NA
3 to 4	3	12	to	15	12.33 ± 1.15
>4	4	14	to	17	22.88 ± 3.01

At Developmental stage 4 inflorescences emerge from ruptured prophyll and peduncular bract. Florets emerge from behind floral bracts for anthesis at approximately leaf stage 16-17. Soon after the receptive window for pollination, the inflorescence underwent necrosis and shed away (Plate 6.9).

6.3.4. Characterisation of Hermaphrodite Inflorescences

Oil palm occasionally produces mixed sex/hermaphrodite inflorescences usually at the transition between the male and female cycles. The hermaphrodite inflorescences encountered had two different morphologies (plate 6.10). The first had a structure that resembles male inflorescence architecture. However, there were truncated female spikelets at the base of the inflorescence axis. This morphology was found associated with the transition from the female phase to the male phase. The second type of hermaphrodite inflorescence had a bunch like structure similar to female inflorescence architecture but with a single male spikelet. This morphology was found associated with the transition from the male phase to the female phase. The male spikelet was found to enter anthesis before the female spikelets of the same inflorescence (plate 6.2D).

6.3.5. Effect of Phenotype on Inflorescence Length

In the initial analyses of field sampling data (Chapter 5), and further analyses of inflorescence length with respect to the established developmental categories (Table 6.3) phenotype (Mantled/Normal) showed a significant effect on the length of inflorescence, within clones. To examine this relationship Analysis of Variance was done on developmental stages Young Triad 1 (YT1) to Mature Inflorescence (MF) excluding the imperfect (incomplete due to nature of sampling) categories Young Bunch (YB) and

Young Inflorescence (YI). The accumulated analysis of variance showed significant three-way interaction between clone, developmental stage and phenotype (Table 6.6, Figure 6.5). However, the effect was highly variable. In mature inflorescences, two clones out of three with comparable samples had higher average length for normal inflorescences compared to mantled. At floral triad stage 4, three out of the four clones had higher average length for normal. Only two clones had comparable samples and one of them had higher average length for normal and the other for mantled.

Table 6.6 Accumulated Analysis of Variance of Sample Data. Variations due to clonal origin, developmental stage, and phenotype (mantled/normal) were analysed. Significant effects are denoted with *. DvlpStage stands for Developmental stage (MF, FT4, FT3, YT2 and YT1, YB and YI were excluded)

Source of variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F value	F probability
Clone	3	7273.74	2424.58	409.68	<.001**
Developmental stage	4	15144.51	3786.13	639.74	<.001**
Phenotype	1	4.76	4.76	0.80	0.37
Clone*DvlpStage	12	2006.33	167.19	28.25	<.001**
Clone*Phenotype	3	17.89	5.96	1.01	0.39
DvlpStage*Phenotype	4	21.59	5.40	0.91	0.46
Clone*DvlpStage*Phenotype	8	113.67	14.21	2.40	0.02*
Residual	93	550.40	5.92		
Total	128	25132.88	196.35		

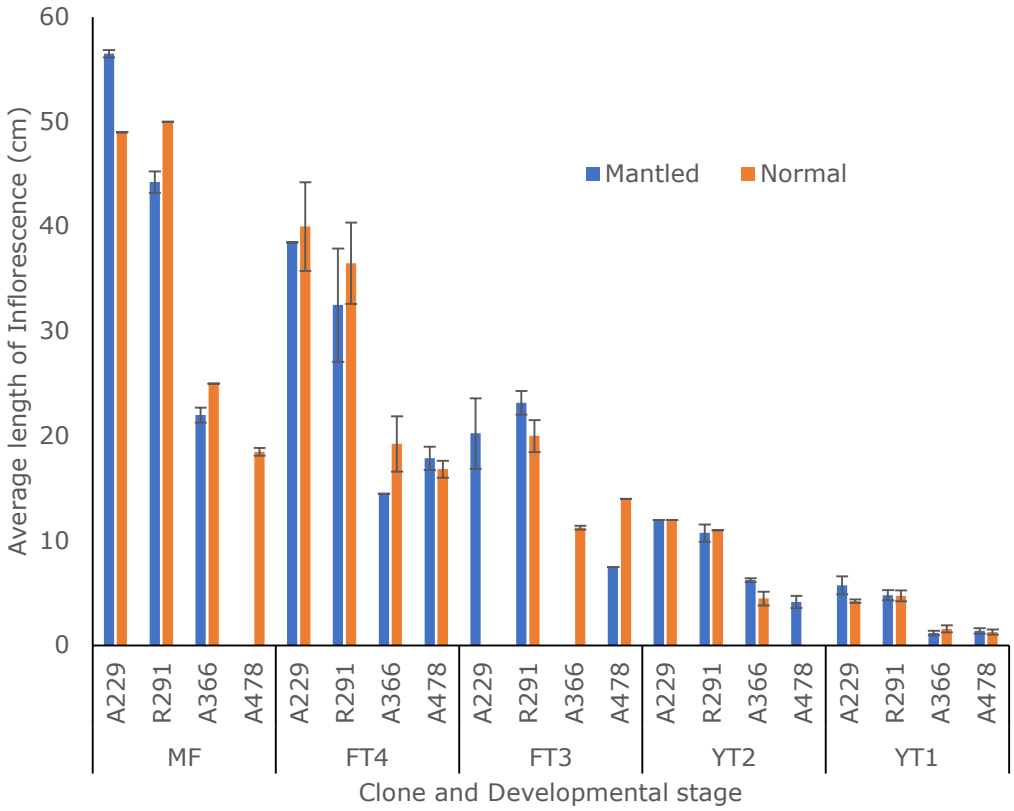

Figure 6.5 Effect of Mantled on Inflorescence Length within Developmental Categories. The Average length of inflorescences of different phenotypes (mantled/normal) is shown within clones at the different developmental stages. Error bars show standard deviation associated with the mean data.

At young triad stages 2 and 1, the difference in average length between mantled and normal was less than 2cm but considering the lengths of inflorescence at these stages, even a small difference may not be negligible. However, the pattern was not consistent. While in one clone, there was no difference, two others had longer mantled inflorescence and three longer normal inflorescences. In total eight comparisons within the same clone at the same developmental stage showed higher average length in normal whereas seven the opposite, the remaining one had equally long normal and mantled samples. The capricious effect of phenotype seen is the possible result of low sample size.

6.4. SUMMARY

Individual inflorescences take 2 to 3 years from floral initiation to maturity. To compare the reproductive development of normal and mantled phenotypes, equivalent samples were required from different stages of the developmental process. The developmental stages extractable through resolute sampling may be classified into 8 developmental categories using the developmental stage prediction algorithm (Sarpan *et al*., 2015) and visual staging proposed here. Further categorisation of young inflorescences was possible through histology. The developmental categories thus ascertained were found to be a more accurate reference for identifying comparable samples.

Developmental classification proposed could also be applied to male inflorescences, to a limited extend. Even though they have a different morphology to the female inflorescences, their developmental pattern was similar. The potential effect of mantled phenotype on the length of inflorescence was analysed for the current data set. However, considering the inconsistency of the relationship and the limited number of samples in the different categories this was probably coincidental.

The developmental classification was suitable for accurate identification of inflorescence developmental stages of oil palm inflorescences. This holds true across different clones and age groups. Within the respective age groups, length of inflorescence was significantly different between the developmental categories. Further, through the robust characterisation carried out here, and further histological study described in chapter 7 it was also possible to use the developmental categories to identify the developmental events in the reproductive developmental process.

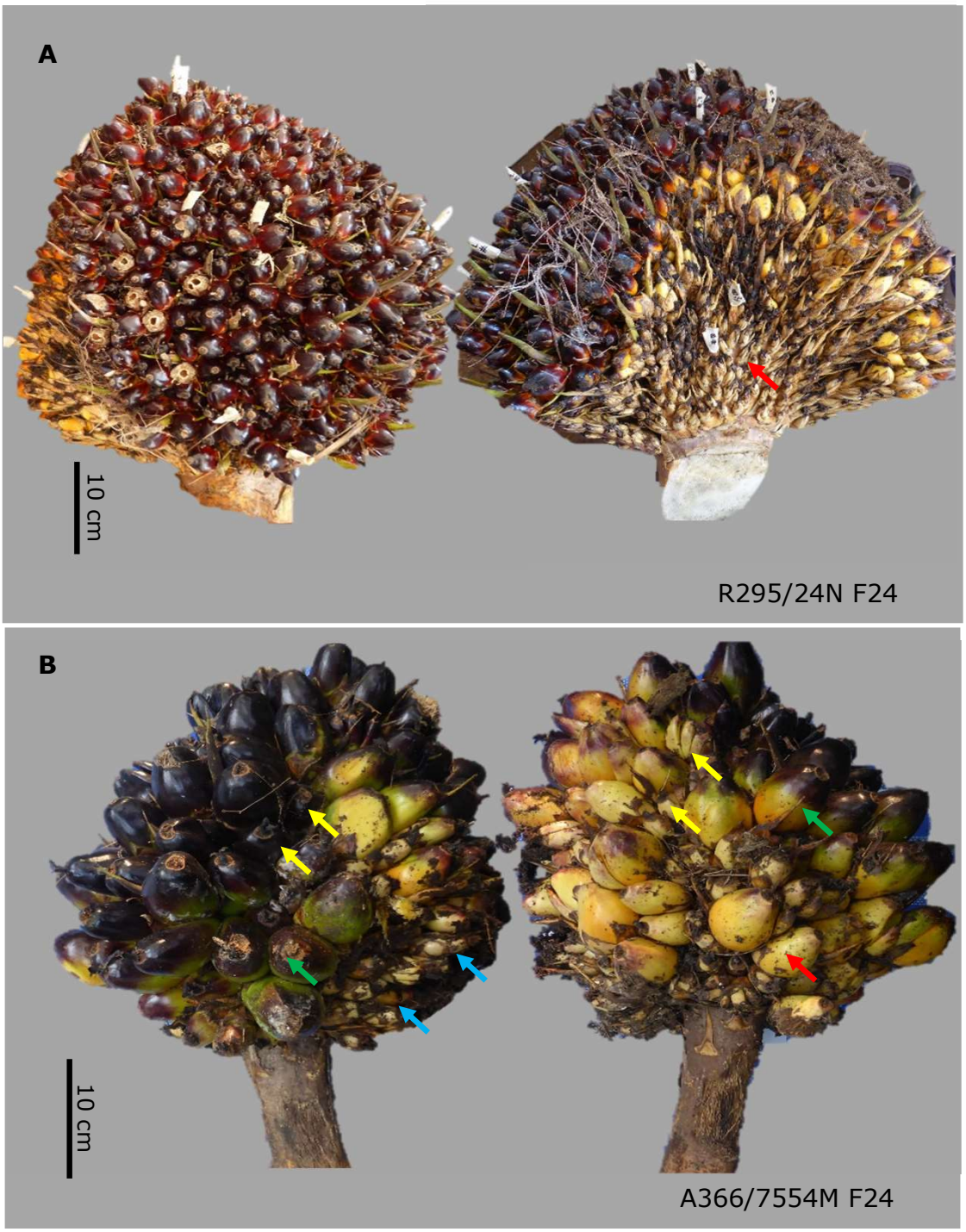

Plate 6.1. Unripe Fruit Bunches.
(A) Anterior (left) and posterior (right) views of a normal unripe fruit bunch from a mature clone. (B) Anterior (left) and posterior (right) views of a less severely mantled unripe fruit bunch from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 10 cm.
Fruit development is observable in the bunches. The spathes no longer enclose the bunch and fruits show almost 100% pigmentation. The stage is characterised by dry necrotic stigmatic lobes and developing kernels in fruits. Unripe fruit bunches are significantly bigger in size in mature clones. Less pigmentation and smaller unfertilized fruits are seen on the posterior side (red arrows) which is pressed against the leaf base, especially in the case of bigger bunches of mature clones. In the less severely mantled bunch (B) fertile fruits with developing kernel (green arrows) and parthenocarpic fruits (blue arrows) are visible. Pseudocarpels are seen like fringelike projections around the central fruit (yellow arrows). Visual scoring of unripe bunches was used for detailed phenotyping of palms (chapter 3, 4).

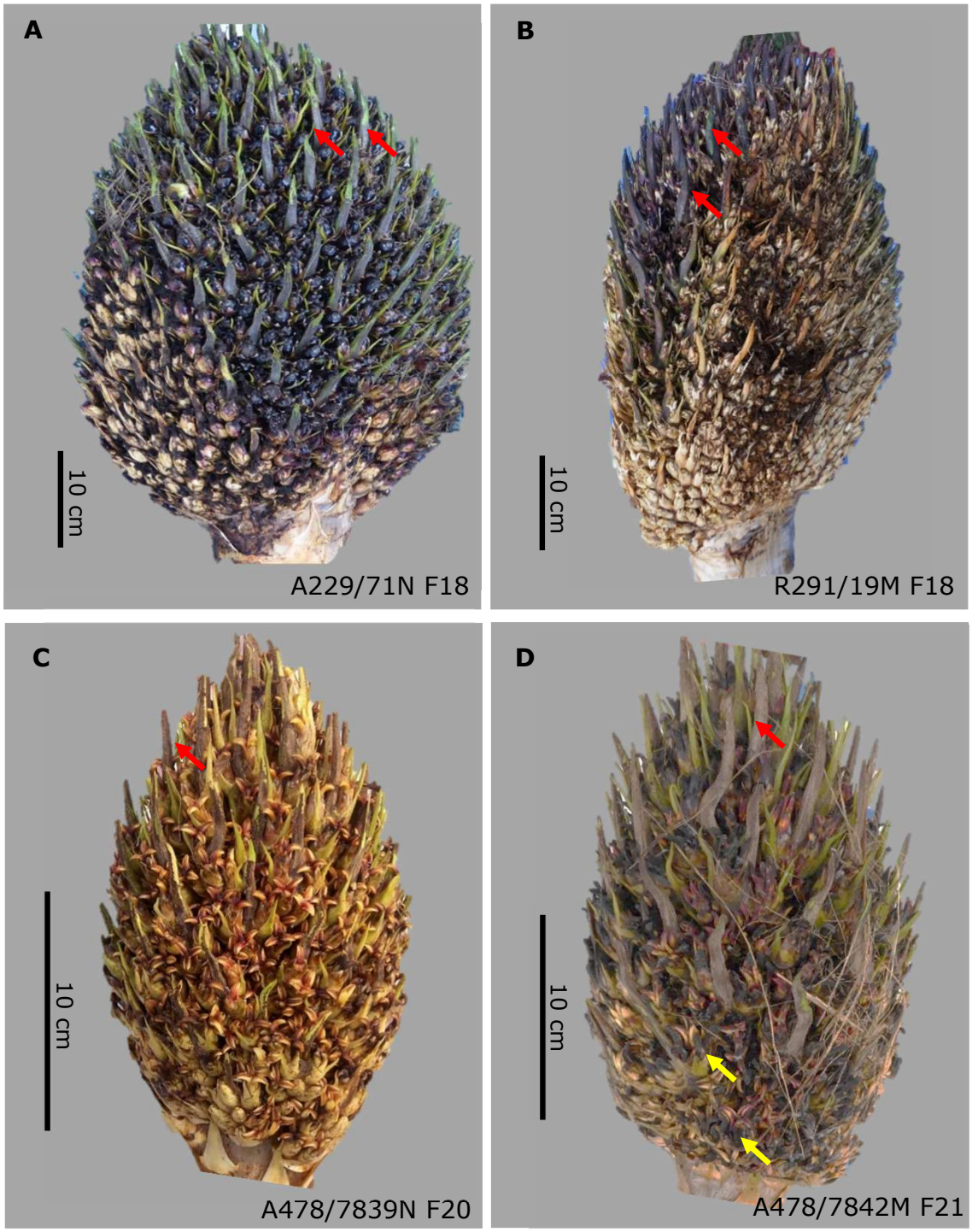

Plate 6.2. Young Bunches.
(A) Normal young bunch from a mature clone. (B) Mantled young bunch from a mature clone. (C) Normal young bunch from a young clone. (D) Mantled young bunch from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 10 cm.
This post anthesis stage is characterised by flowers/fruits with pigmented tepals and stigmatic lobes are gaping and past the receptive stage. But Kernel development is not yet clearly evident. Spathes are no longer enclosing the bunch at this stage. Floral bracts (red arrows) that are fibrous by this stage give a characteristic spiky appearance. Pseudocarpels (yellow arrow) are visible surrounding the functional carpels in the mantled bunches. Young bunches of younger clones (C, D) are smaller compared to those from mature clones (A, B). Within the age category clones had similar sized bunches, irrespective of phenotype.

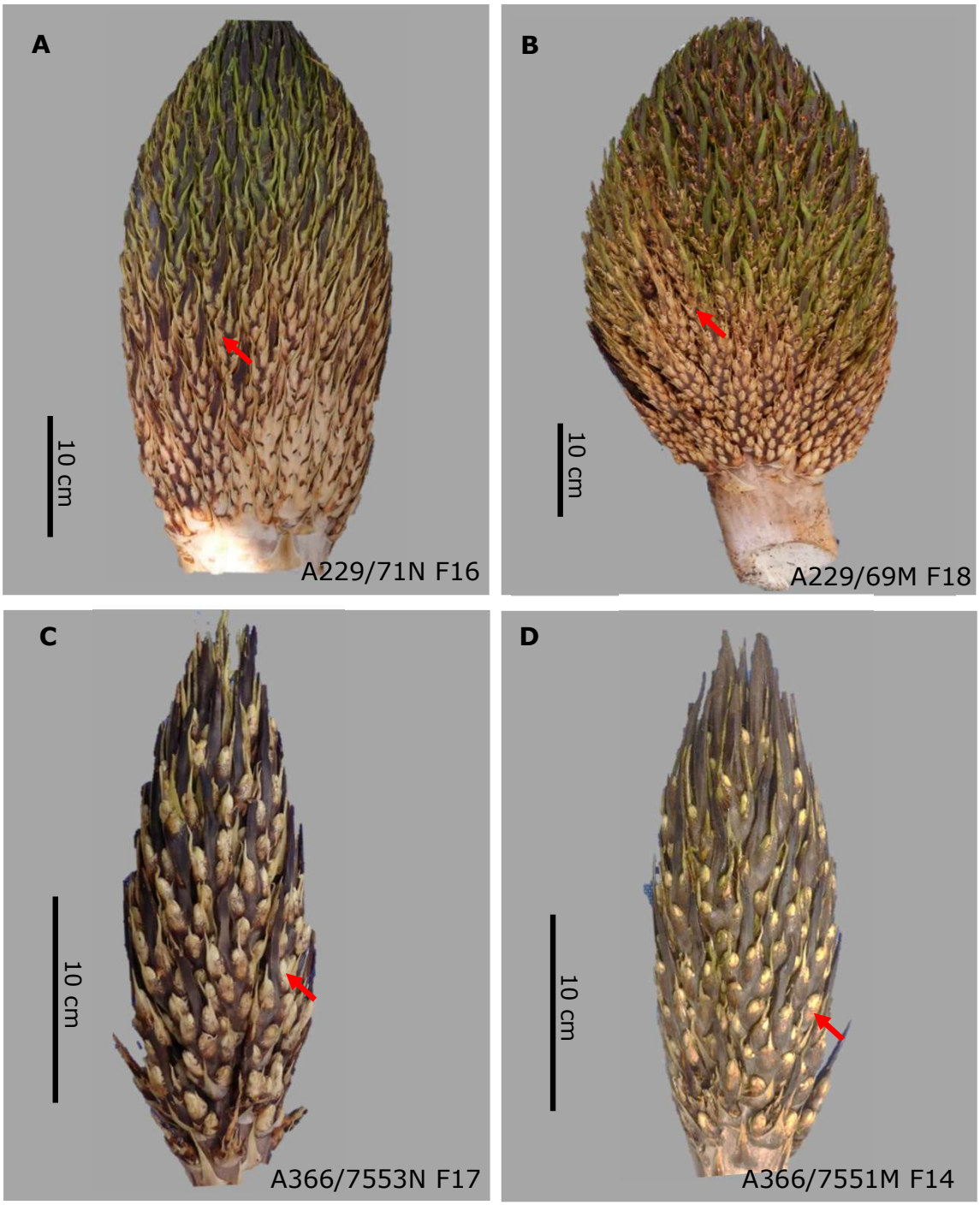

Plate 6.3. Mature Inflorescences.
(A) Normal mature inflorescence from a mature clone. (B) Mantled mature inflorescences from a mature clone. (C) Normal mature inflorescence from a young clone. (D) Mantled mature inflorescence from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 10 cm.
Predictive algorithm places this at a stage >4. At this stage inflorescences emerges from ruptured spathes (prophyll and peduncular bract) and develop more than 50% pigmentation, predominantly on the anterior side exposed to sunlight. Individual flowers have pigmented tepals and are emerging from behind floral bracts (red arrows). The Abortive Staminate Flowers (ASFs) are already shed. Mature gaping stigma is seen at anthesis. Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B).

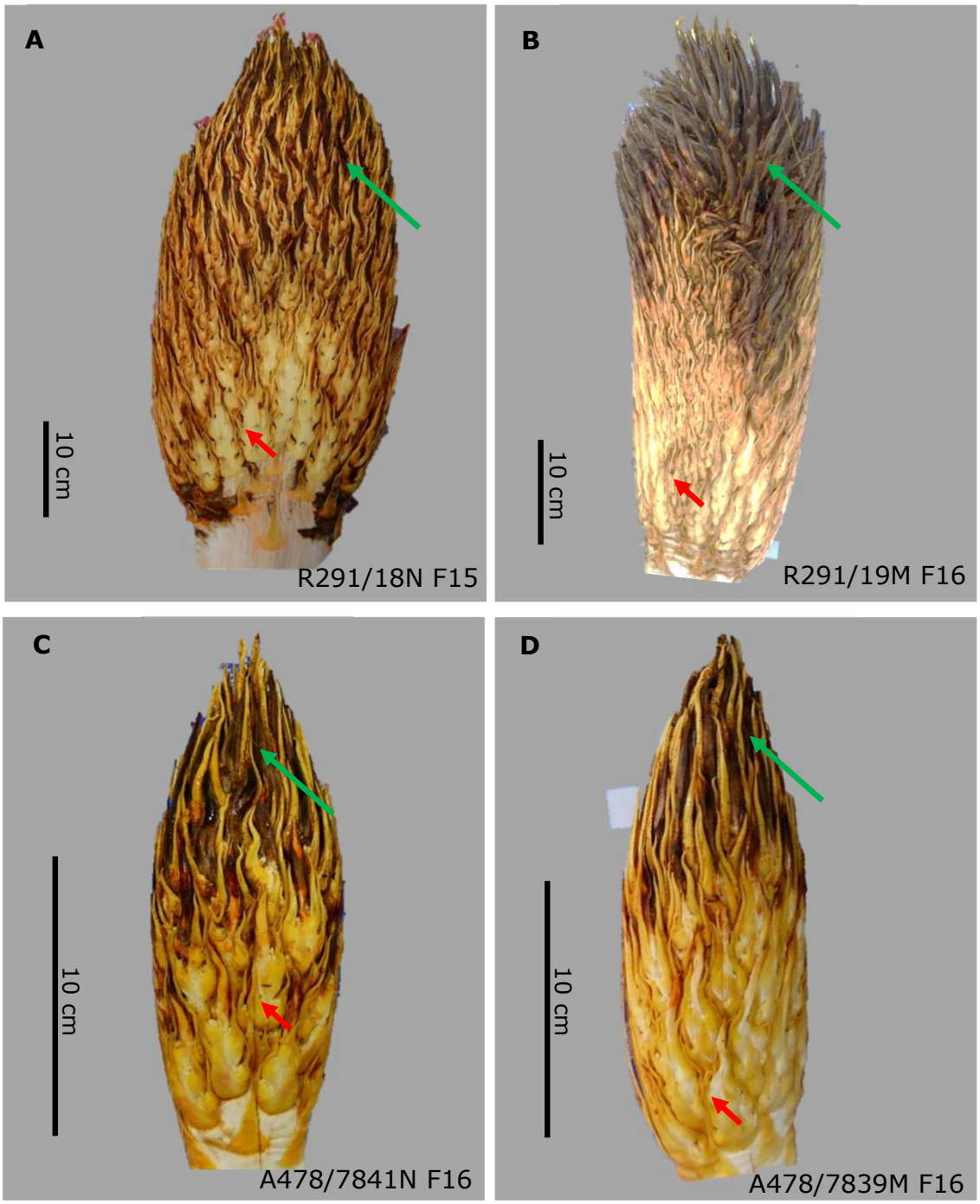

Plate 6.4. Stage 4 Floral Triads.
(A) Normal stage 4 floral triads from a mature clone. (B) Mantled stage 4 floral triads from a mature clone. (A) Normal stage 4 floral triads from a young clone. (D) Mantled stage 4 floral triads from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 10 cm.
Predictive algorithm places this at stage 4. At this stage of development the prophyll is torn and the inflorescence show certain degree of pigmentation (green arrows). Growth spurt evident in the floral triads. The middle pistillate flowers (red arrows) are larger than the two adhering ASFs and is starting to emerge from under the floral bract. Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B).

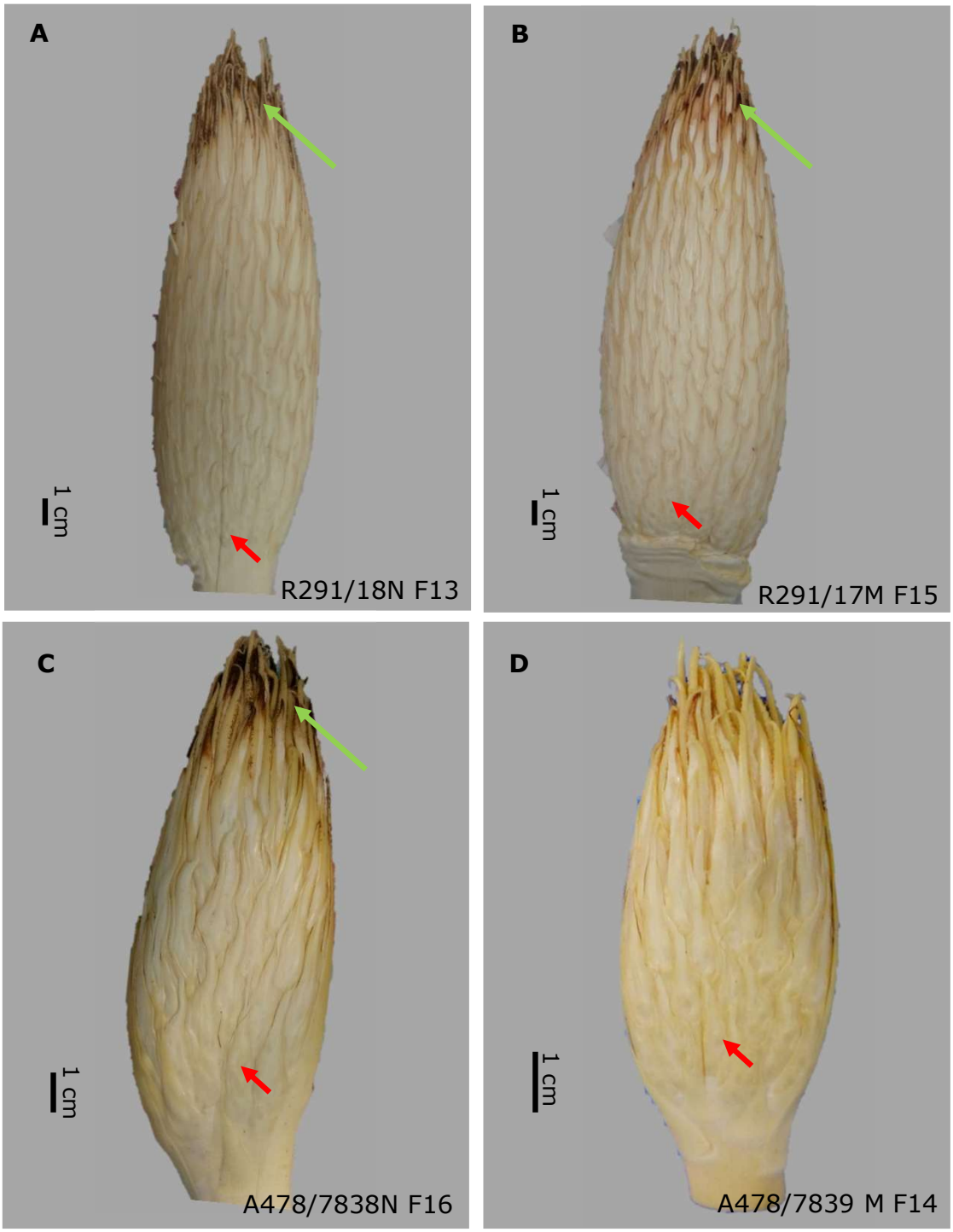

Plate 6.5. Stage 3 Floral Triads.
(A) Normal stage 3 floral triads from a mature clone. (B) Mantled stage 3 floral triads from a mature clone. (A) Normal stage 3 floral triads from a young clone. (D) Mantled stage 3 floral triads from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 1 cm.
Predictive algorithm places this at stage 3. At this stage of development the prophyll is starting to become fibrous and tear. Beginning of pigmentation is visible at the apex of the inflorescences (green arrows). Growth spurt evident in the floral triads. The middle pistillate flowers are larger than the two adhering ASFs and is seen as small bulges under the floral bract(red arrows). Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B).

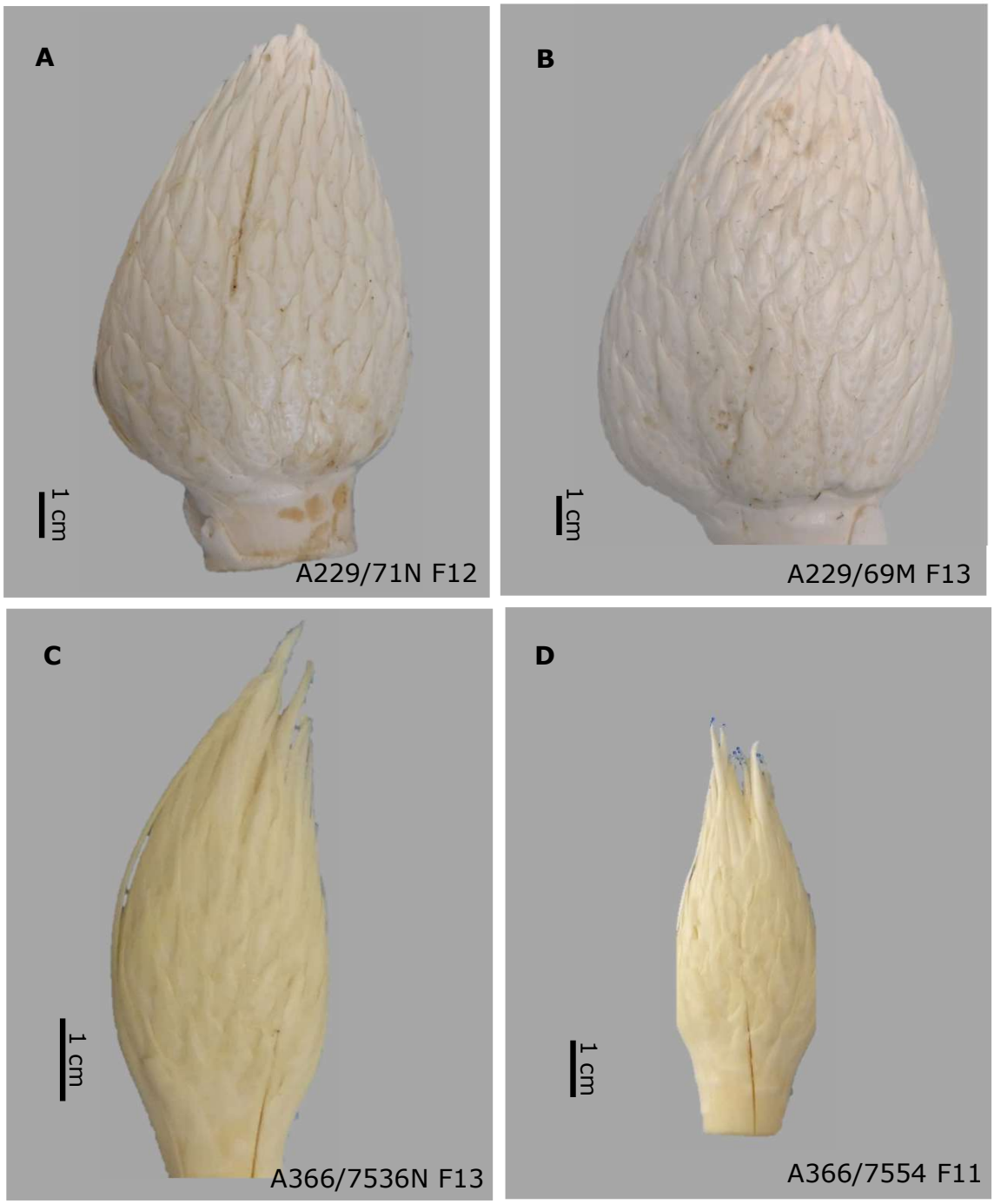

Plate 6.6. Stage 2 Young Triads.
(A) Normal stage 2 young triads from a mature clone. (B) Mantled stage 2 young triads from a mature clone. (A) Normal stage 2 young triads from a young clone. (D) Mantled stage 2 young triads from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 1 cm.
Predictive algorithm places this at stage 2. At this stage of development the inflorescences are found fully enclosed within the protective bracts and show no pigmentation, they are pale yellow to white in colour. Spikelets carry floral triads still enclosed by the floral bract. As this is before the growth spurt or at the start of it, all three florets of the triad are approximately the same size. Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B), but the difference is less pronounced.

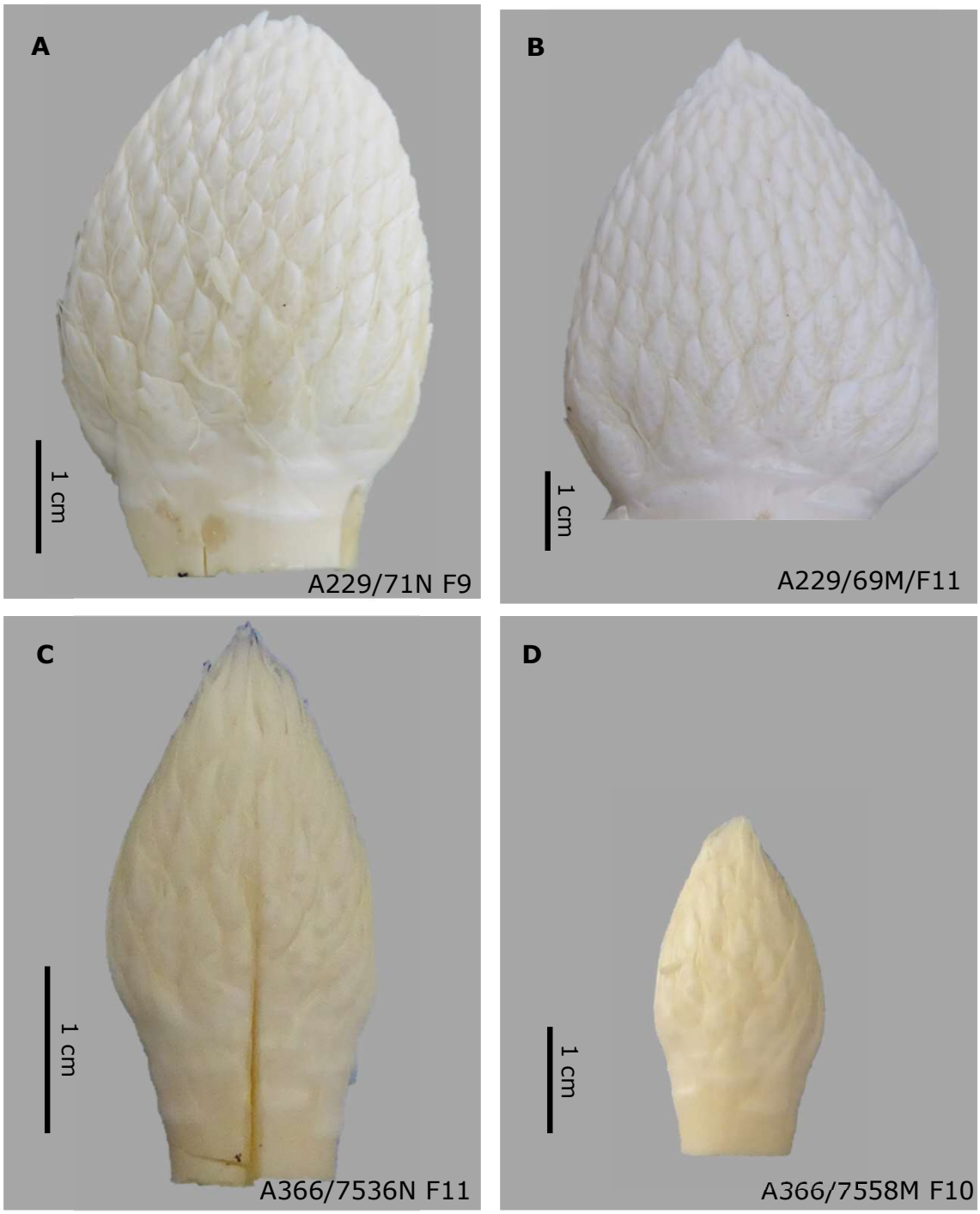

Plate 6.7. Stage 1 Young Triads.
(A) Normal stage 1 young triads from a mature clone. (B) Mantled stage 1 young triads from a mature clone. (A) Normal stage 1 young triads from a young clone. (D) Mantled stage 1 young triads from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 1 cm.
Predictive algorithm places this at stage 1. At this stage of development the inflorescences are found fully enclosed within the protective bracts and show no pigmentation, they are pale yellow to white in colour. Spikelets carry floral triads still enclosed by the floral bract. As this is before the growth spurt or at the start of it, all three florets of the triad are approximately the same size. Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B), but the difference is less pronounced.

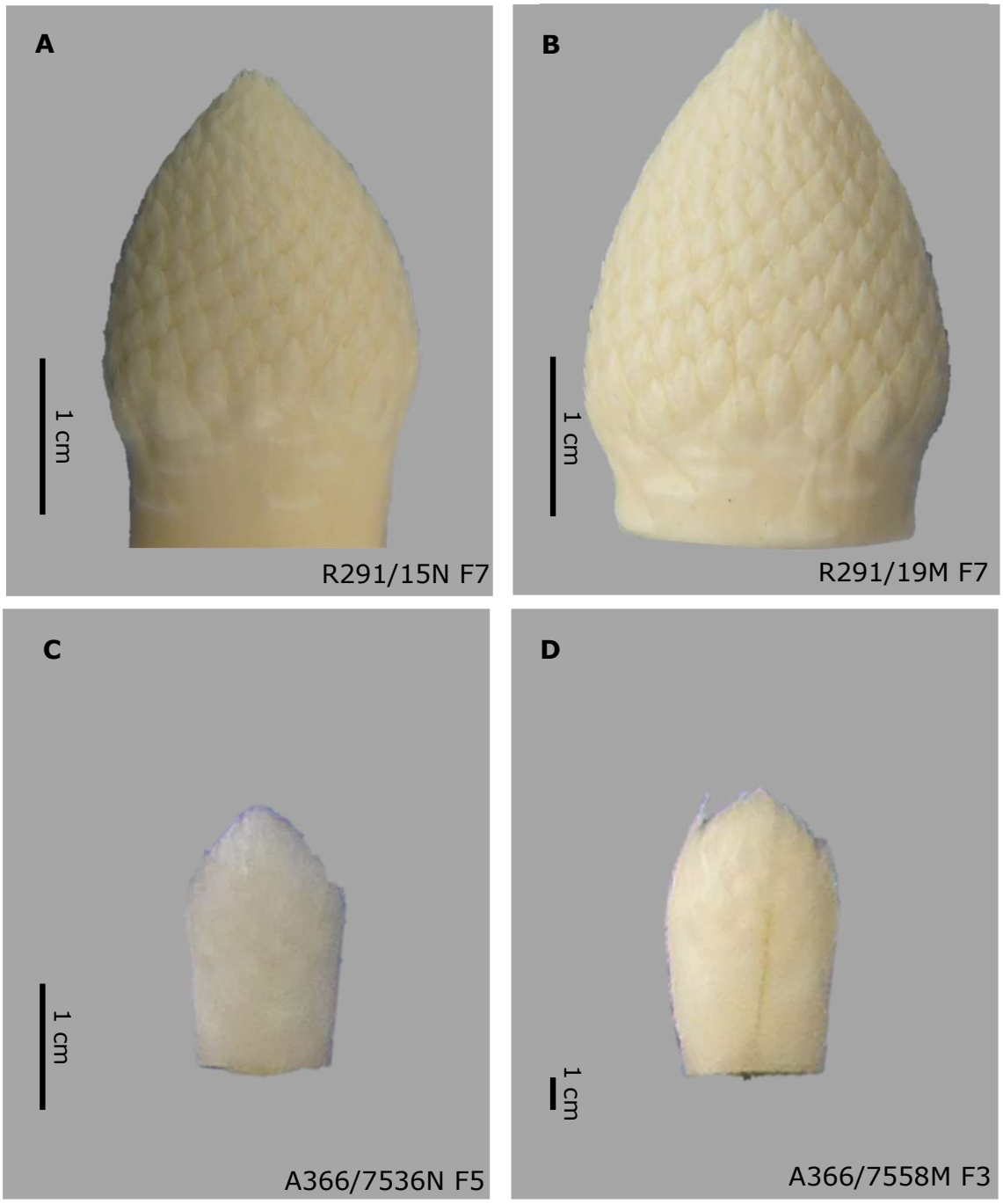

Plate 6.8 Young inflorescences.
(A) Normal young inflorescence from a mature clone. (B) Mantled young inflorescence from a mature clone. (A) Normal stage young inflorescence from a young clone. (D) Mantled young inflorescence from a young clone. Sample IDs includes clone name, palm number, phenotype (N-normal, M- mantled) and leaf stage. The bars represent 1 cm.
Predictive algorithm places this at stage <1. At this stage of development the inflorescences are found fully enclosed within the protective bracts and show no pigmentation, they are pale yellow to white in colour. Floral triads of this stage are not distinguishable by naked eye. Further developmental classification of young inflorescence was possible through histology. Inflorescences from young clones (C, D) are smaller than those from mature clones (A, B), but the difference is less pronounced.

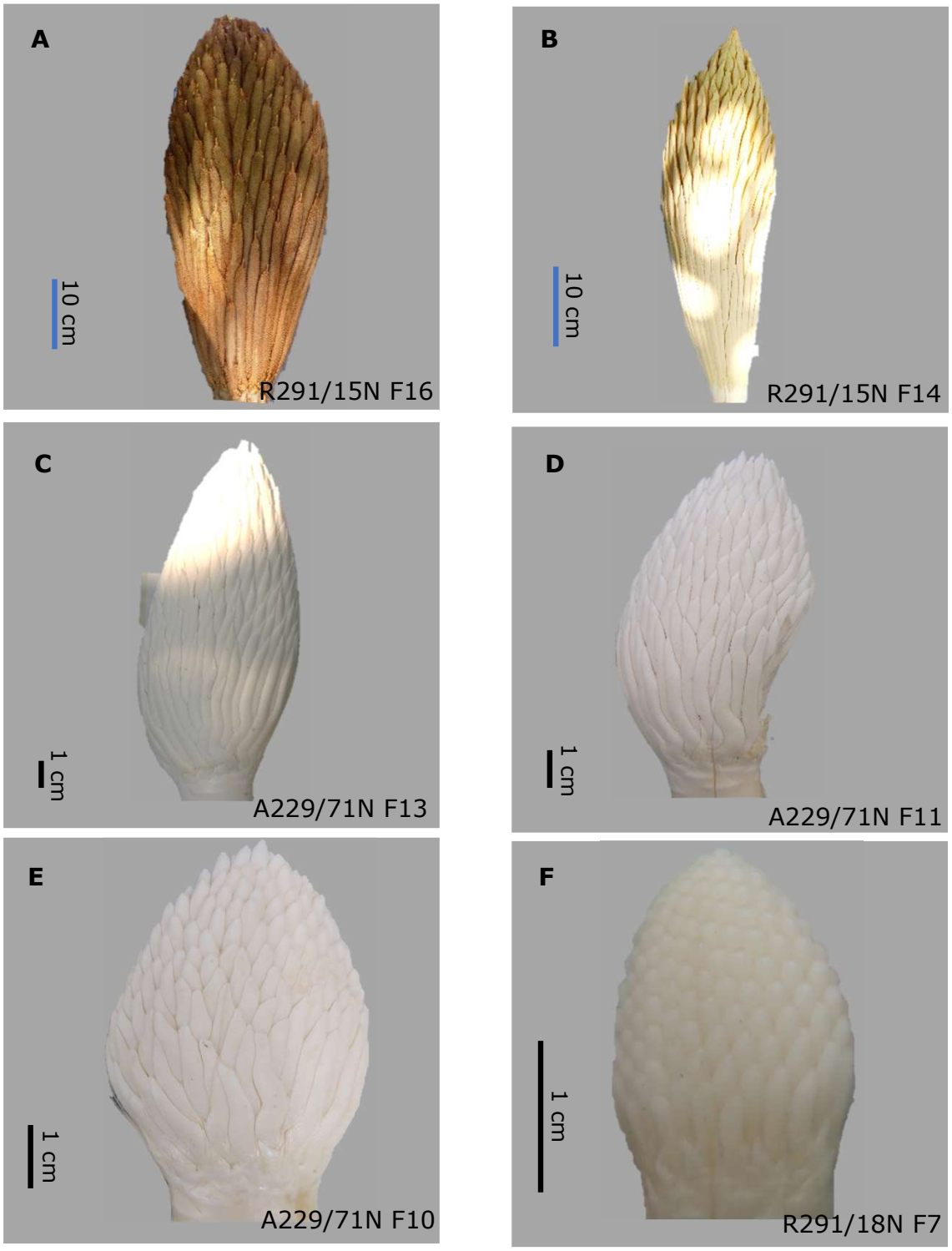

Plate 6.9. Male Inflorescences at Different Stages of Development.
(A) Mature inflorescence prior to anthesis at stage >4. (B) Mature inflorescence at stage 4. (C) Developing inflorescence at stage 3. (D) Developing inflorescence at stage 2. (E) Developing inflorescence at stage 1. (F) Young inflorescence at stage <1. Sample IDs includes clone name, palm number, phenotype (N-normal, M-mantled) and leaf stage. The blue bars in A, B represents 10 cm and the black bars in C to F represents 1 cm.
Categorisation of male inflorescences was based on predictive algorithm alone. Stages closer to anthesis (A) showed pigmentation. Until developmental stage 4, un-emerged inflorescences with zero pigmentation were observed (C to F) . The individual functional staminate flowers are protected by floral bracts. A gradual increase in length at initial stages (from F backwords to C) followed by an exponential increase in length from developmental stage 3 (from C, coinciding with organogenesis) is observed.

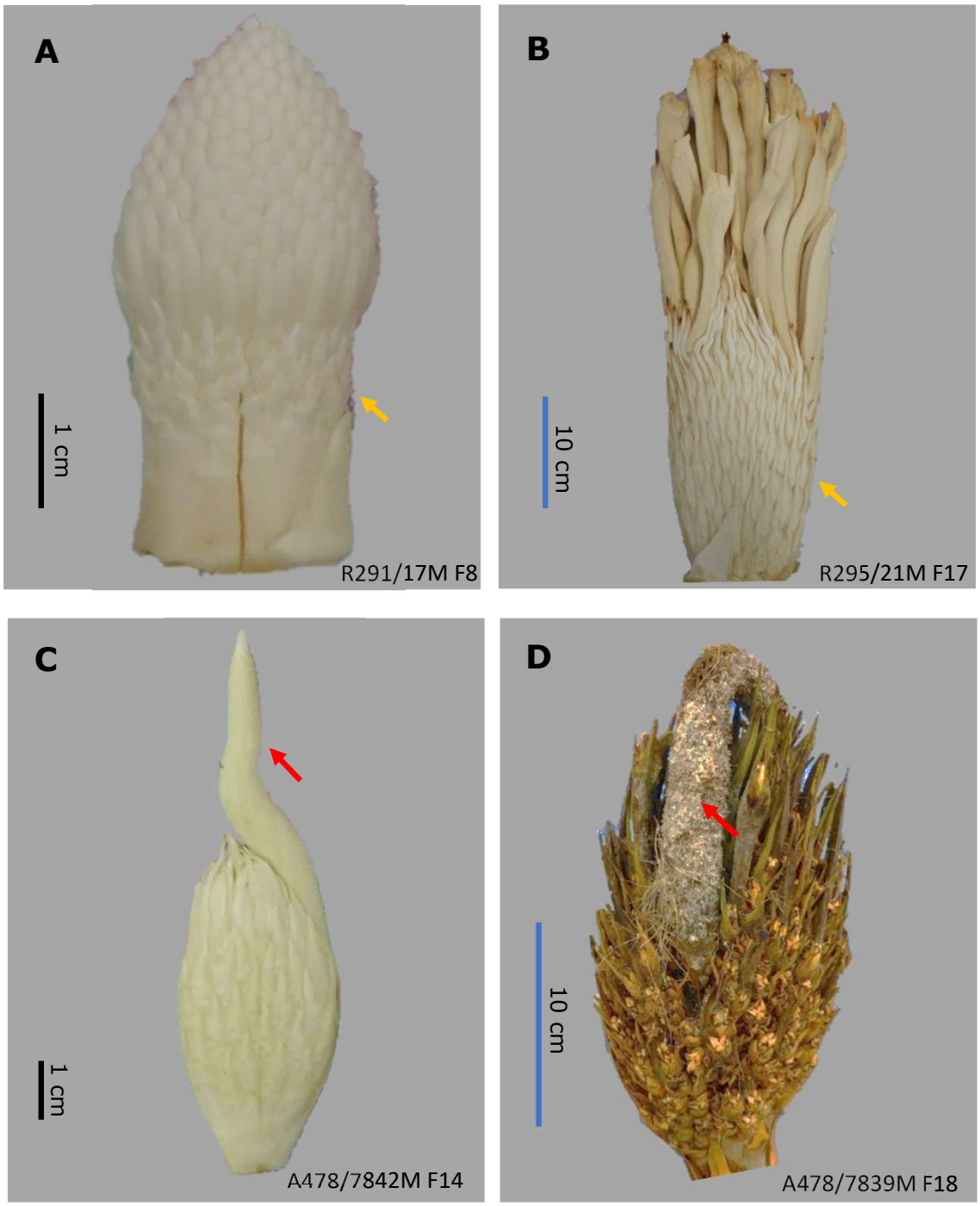

Plate 6.10. Different Morphologies of Hermaphrodite Inflorescences.
Sample IDs includes clone name, palm number, phenotype (N-normal, M-mantled) and leaf stage. The blue bars in A, C represents 10cm and the black bars in C, D represents 1 cm.
Two different morphologies were observed. The first (A, B) a structure that resembles male inflorescence architecture but with female spikelets (yellow arrows) at the base of the inflorescence axis. This was found associated with the transition from female phase to male phase. The second (C, D) a bunch like structure similar to female inflorescence architecture but with a single male spikelet (red arrows). This was found associated with the transition from male phase to female phase. The male spikelet was found to enter anthesis before the female spikelets of the same inflorescence (D).

CHAPTER 7
COMPARATIVE REPRODUCTIVE DEVELOPMENTAL SERIES FOR OIL PALM

7.1. INTRODUCTION

Objectives:

- Histological analysis of oil palm inflorescence samples to construct a detailed description of the developmental stages in the form of a reproductive developmental series and to compare normal and mantled reproductive development

Within the crown of an oil palm, the single shoot apical meristem is enveloped in developing leaves, according to Combres *et al*., (2013) about 60 in number. Then Outside about 40 autotrophic expanded leaves are visible. At the base of these leaves, both within and outside the crown of the palm, is where the inflorescence develops. Oil palm inflorescence is a complex spadix with two degrees of branching- the rachis gives rise to spikelets, and the spikelets bear the functional staminate or pistillate flowers in male and female inflorescences respectively (Adam *et al*., 2005). One palm produces between 20 and 30 new expanded leaves and thus new inflorescences per year. So, at any given movement an adult palm carries approximately 50 inflorescences at different stages of development, in addition to multiple ripening bunches (Combres *et al*., 2013).

For systematic analysis of reproductive development in normal and mantled oil palm, inflorescence samples collected were classified into distinct developmental stages. These developmental stages were characterised using the predictive algorithm (Sarpan *et al*., 2015) and visual staging as

detailed in the Chapter 6. Visual staging primarily utilised macro features visible to the naked eye, and observable in the field. As part of this research a detailed histological analysis of oil palm inflorescence samples was carried out. The histology protocol was adapted from previous literature (Adam *et al*., 2005, Sarpan *et al*., 2015) and was optimised through trials (Chapter 5). High resolution microscopy helped to pinpoint morphological and physiological changes during inflorescence development at the cellular level.

Developmental series prepared based on the analysis of multiple clones is provided herewith. This developmental series enables comparison within and between clones and between normal and mantled phenotypes. The mantled phenotype was studied in detail throughout the entire developmental process alongside equivalent samples from normal palms of the same clone which has been never done before. However, this project focussed on the characterisation of the aberrant phenotype rather than weigh in on the causality.

Using microscopic techniques, the earliest stage of development where mantled phenotype was identified. Moreover, Key differences between normal and mantled reproductive development were visualised. The results generated adds on to the knowledge base and the methods proposed can be used to systematise future studies in this field. Some of the observations made were in contradiction to previous reports and are discussed below.

Lastly, the results were compared to published descriptions of reproductive development in closely related palms namely, American oil palm, *Elaeis oleifera* and coconut palm, *Cocos nucifera*.

7.2. MATERIALS & METHODS

See Chapter 3, section 3.2.1 for details of plant material and sample types. Genotyping results are available in Appendix 2, phenotyping data in Appendix 3 and field sampling data in Appendix 6.

Non-destructive sampling (F7 to F18) was conducted in 8 mature palms (Ten years of age) belonging to 2 clones (A229 and R291) and destructive sampling (F-29 to F21) in 9 young palms (Three years of age) from 2 clones (A366 and A478) as described in Chapter 5, sections 5.2.1 and 5.2.2.

The samples collected were categorised into 8 major categories based on the developmental classification established in chapter 6. Further classification of young inflorescence was done through histology.

Comparative Normal and Mantled samples were selected from sampling groups for the preparation of the developmental series. Microscopic analysis of the samples was done following the histology techniques described in chapter 5, sections 5.2.6 to 5.2.8.

7.3. RESULTS AND DISCUSSION

7.3.1. Chronology of Developmental Events in Reproductive Development of Oil palm

Through the sampling method used in this study it was possible to obtain inflorescence samples as young as F-29 (30 ranks within the crown of the palm). By histological approaches adopted, it was possible to analyse samples up to anthesis, after anthesis the tissue was too hard for sectioning.

In the clones included in the current study anthesis or flowering was found to occur roughly at F18 to F20. This was approximately 9 months after emergence of the subtending leaf from the crown. Two thirds of the developmental period was required for expansion of the inflorescence meristem to form the spathes, spikelet bracts, spikelets and floral bracts. This was found to be a slow process. Sex differentiation was estimated to occur either between F-5 and F–10, coinciding with spikelet bract initiation. However, in the current sample set identification of sex was possible through histological analysis only at F2, where arrangement of spikelets were clearly visible.

The last one third of the developmental period, starting roughly at F4 to F7 was involved in the development of the individual flowers. Organogenesis, that is the formation of individual floral organs was found to occur roughly at F7 and was associated with rapid expansion of inflorescences. Chronology of events in the reproductive development of oil palm is summarised in Figure 7.1. The findings corroborated previous reports by Adam *et al* (2005) and Corley and Tinker (2008) on reproductive development in seed derived oil palms.

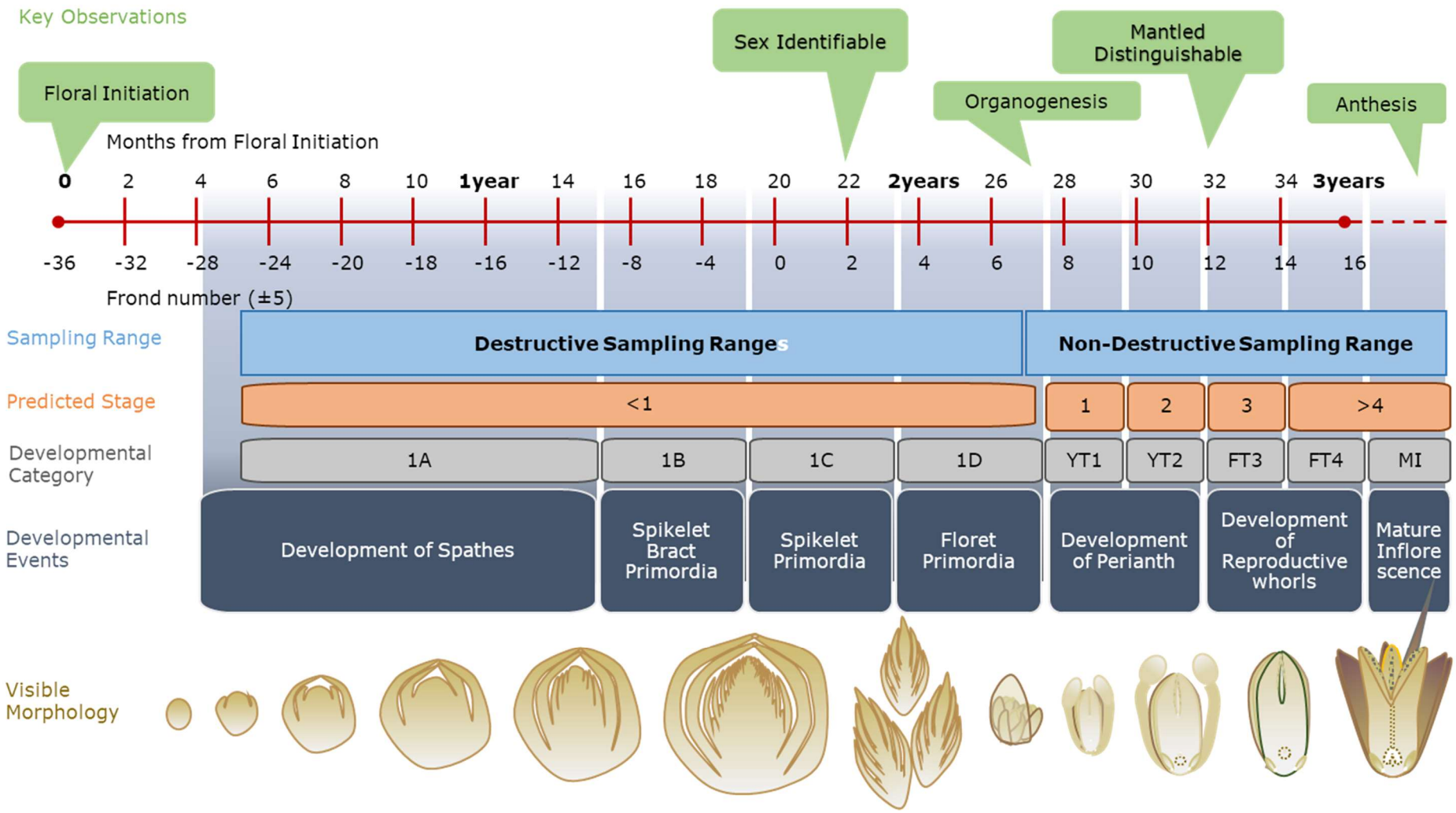

Figure 7.1 Chronological Depiction of Developmental Events in Oil Palm Reproductive Development. Time line shows months from floral initiation on top and approximate frond number on the bottom. The different rows represent key observations at different points of time indicated in green, sampling ranges in blue, predicted stage according to the predictive algorithm in orange, the developmental categories established in grey, developmental events in blue and finally illustrations of visible morphology in a developing female inflorescence at the bottom. Morphology of developing inflorescence, spikelets, floral triads and finally a mature pistillate flower are depicted (Original Illustration based on research findings).

7.3.2. Reproductive Developmental Series

Comparative Normal and Mantled samples were selected from sampling groups for the preparation of the developmental series. The different elements of the developmental series are summarised in Table 7.1. Predicted stage according to the predictive algorithm by Sarpan *et al*., (2015) as well as the developmental category/stage following the developmental classification which also considers visual staging and histology, were both used to describe the different elements.

Table 7.1 Elements of the Developmental Series. Different elements of the developmental series and their relationships to the predicted stages and developmental categories are detailed below.

Developmental Series Element	Predicted Stage	Developmental Category
Early Inflorescence Development	<1	Young Inflorescence (YI)
		• 1A development of spathes
		• 1B development of spikelet bract primordia
		• 1C development of spikelet primordia
		• 1D development of floret primordia
Development of Floral Triads (in female inflorescences)	1	Young Triad 1 (YT1)
	2	Young Triad 2 (YT2)
	3	Floral Triad 3 (FT3)
	4	Floral Triad 4 (FT4)
Maturation of Pistillate Flower	>4	Mature Flower(/Inflorescence) (MF)
Staminate Flower Development	2	Young Flower
	>4	Mature Flower

Each of these elements are described in more detail in the following sections. At each stage, the histology of Normal and Mantled samples of the same clone, were compared side by side. Further comparisons were carried

out across clones and across age categories (young and mature) where possible.

7.3.3. Early Development in Normal and Mantled Inflorescences

Young Inflorescences (developmental category 1) represented stages of early inflorescence development. The predictive algorithm placed them all in the development interval <1. Further classification of young inflorescence was not possible by simple observation. However, based on histology, they were further classified as (1A) the development of spathes (Figures 7.2, 7.3), (1B) development of spikelet bract primordia (Figures 7.3, 7.4), (1C) development of spikelet primordia (Figure 7.5) and (1D) development of floret primordia (Figure 7.6). Stained tissue sections at these key stages of early inflorescence development (Figure 7.2, 7.3, 7.4, 7.5 and 7.6) were found to be similar in normal and mantled inflorescences.

(1A) The Development of Spathes: Oil palm inflorescences develop enveloped in two protective spathes namely, the outer spathe or prophyll and the inner spathe or peduncular bract. At leaf stage F-25 in normal and F-20 in mantled inflorescence meristem was seen enveloped by prophyll (Figure 7.2 A, B). The meristem was flanked by the peduncular bract initials laterally.

The meristematic dome displayed zonation of cells (Figure 7.2C). The outermost layer L1 was composed of tightly packed, brick-shaped cells with no spaces between them. Lateral growth was initiated by periclinal division in L1 (Figure 7.2D), followed by anticlinal division which resulted in their expansion. Below L1, layer L2 was the reserve of meristematic cells that were small, round and thin walled with a large nucleus and no vacuoles.

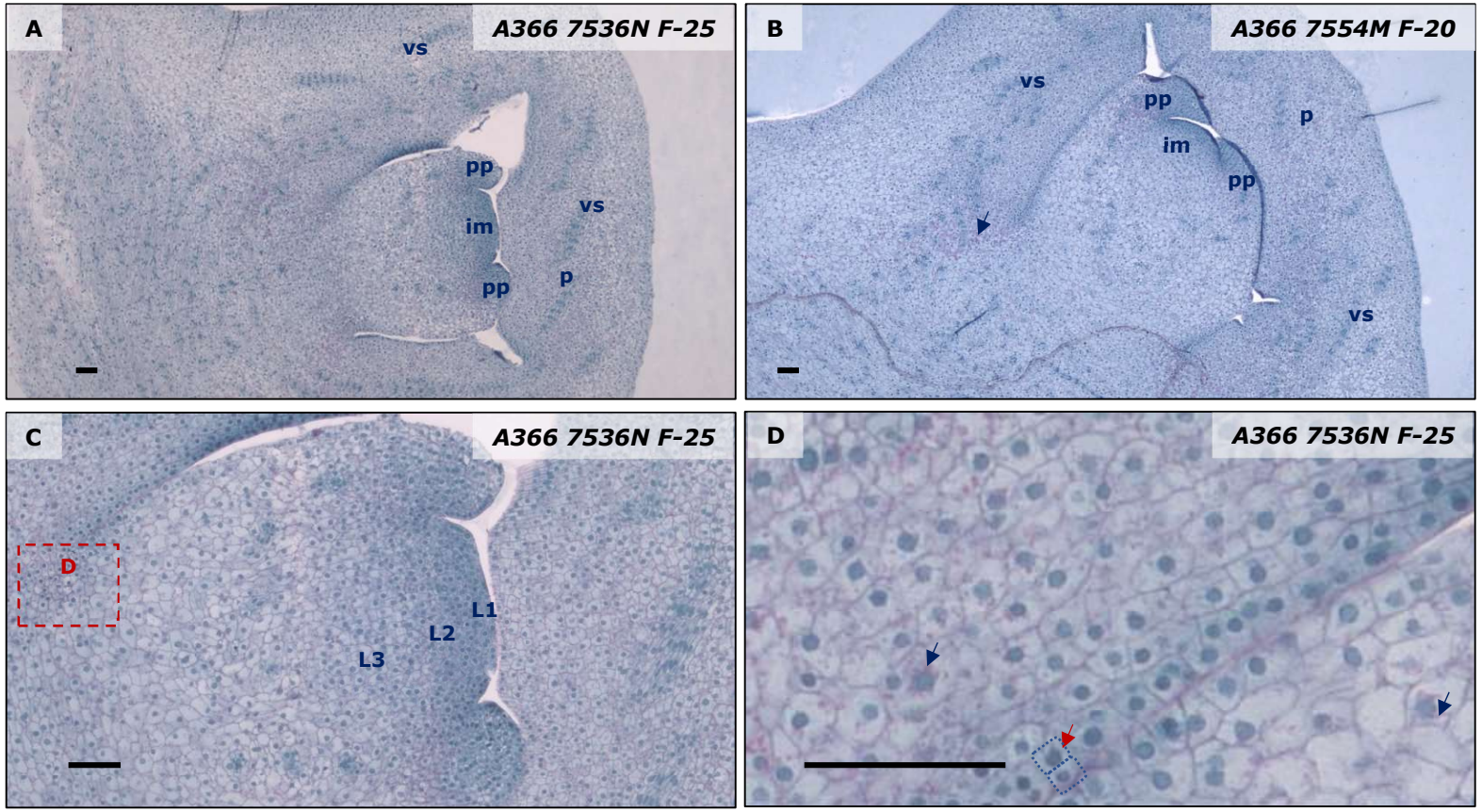

Figure 7.2 Early Inflorescence Development at Developmental Stage 1A. Sections of inflorescence meristems at leaf stage F-25 (A, C, D) in normal and F-20 in mantled (B) showing development of spathes (p, pp). L1, L2 and L3 are showing zonation in cells. Red dashed box in C shows position of D. Blue arrows (B, D) direct to large accumulation of starch indicated by the red staining from periodic acid - Schiff's reaction. Red arrow shows periclinal division in the epidermis (L1). im- inflorescence meristem, p- prophyll, pp- peduncular bract primordia, vs- vascular strands, the bars represent 100 µm.

Beneath these upper layers, L3 had highly vacuolated cells and vascular tissue. Vascular strips were visible in the prophyll, and as indicated by the red stain of periodic acid - Schiff's reaction, there was an accumulation of starch (Figure 7.2 D).

Despite the difference in leaf stage (F-25 of the normal palm was equivalent to F-20 of the mantled palm, mantled palm of the sampling group lagged in development), no differences were observable between normal and mantled samples (Figure 7.2).

(1B) The development of spikelet bract primordia: Similar to the prophyll, the peduncular bract also developed and enveloped the growing inflorescence meristem as seen at leaf stage F-15 in normal and F-14 in mantled (Figure 7.3). At this stage, developmental Stage 1B, the inflorescence meristem maintained a similar form as before but now with the spikelet bracts developing on either side of the meristematic dome (Figure 7.3). The inflorescence meristem was mitotically highly active, as evidenced by the low nuclear to cytoplasm ratio. Periclinal and anticlinal divisions contributed to the growth of inflorescence in length and width.

(1C) The development of spikelet primordia: In the progression to developmental Stage 1C, alongside the elongation of the axis of the rachis, spikelet bracts developed laterally (Figure 7.4 A, B). Initiation of spikelet meristem was visible at F-5 in normal and F-4 in mantled and the middle of the inflorescence axis was highly vascularised at this stage (Figure 7.4 C, D).

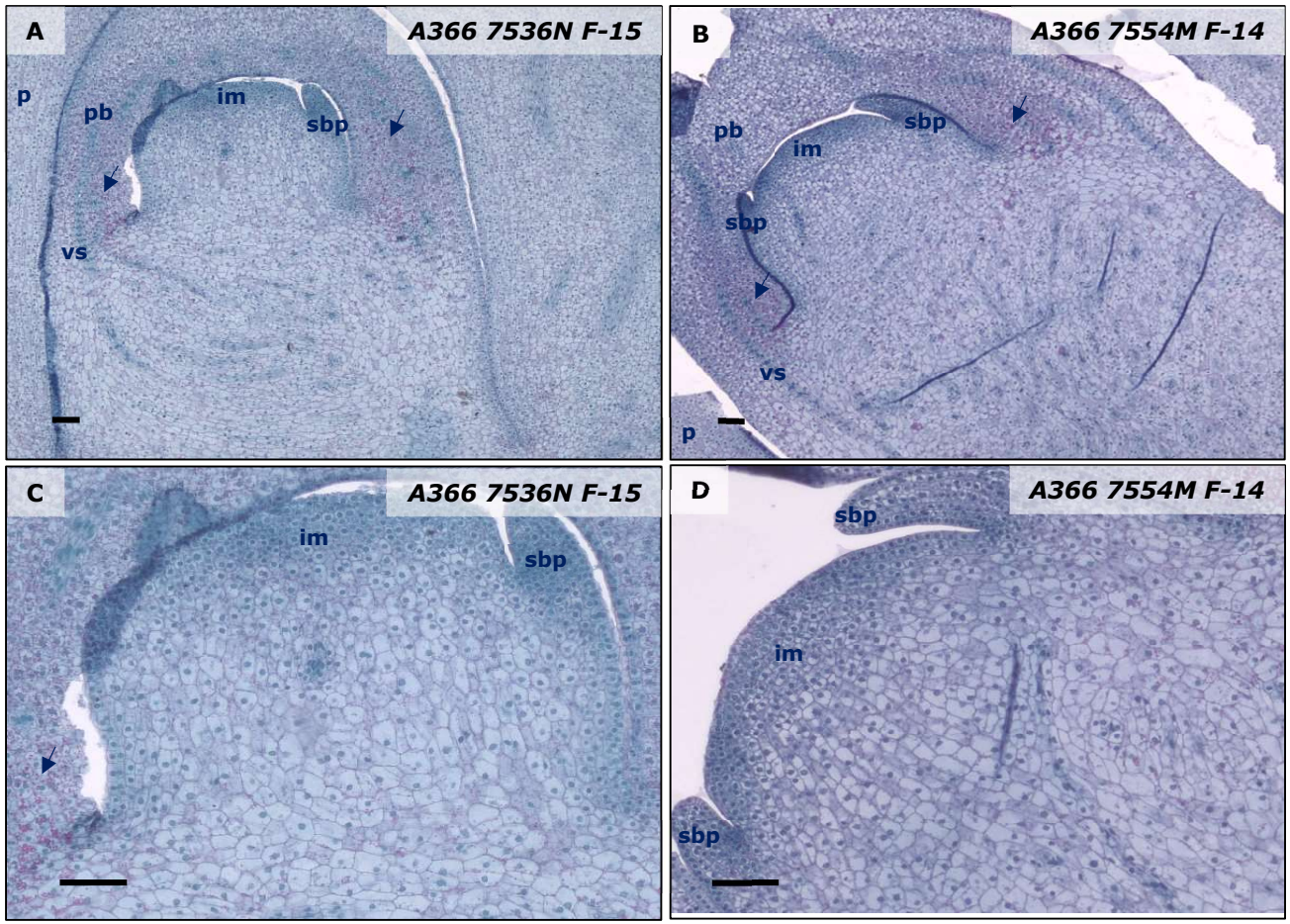

Figure 7.3 Early Inflorescence Development at Developmental Stage 1B. Sections of inflorescence meristems at leaf stage F-15 (A, C) in normal and F-14 in mantled (B, D) showing development of spathes (p, pb) and spikelet bract primordia (sbp). Blue arrows (A, B, C) indicate large accumulation of starch indicated by the red staining from periodic acid - Schiff's reaction. im- inflorescence meristem, p- prophyll, pb- peduncular bract, sbp- spikelet bract primordium, vs- vascular strands. The bar represents 100 µm.

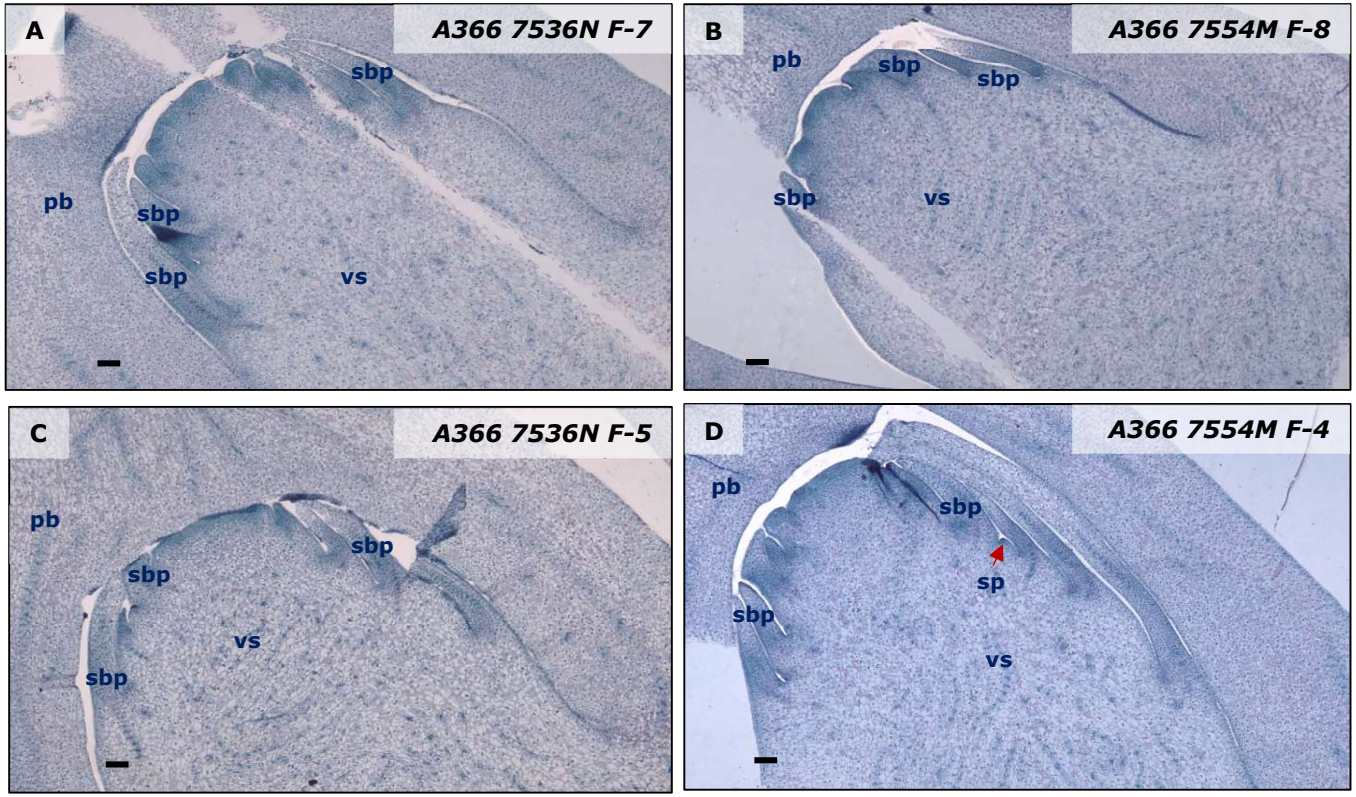

Figure 7.4 Development of Spikelet Bract Primordia and Formation of Spikelet Primordia. Sections of inflorescence meristems at leaf stage F-7 (A), F-5 (C) in normal and F-8 (B), F-4 (D) in mantled are shown. Development of spikelet bract primordia (sbp) is visible at developmental Stage 1B (A, B) and initiation of spikelet meristem (sp) is visible at developmental Stage 1C (C, D). Red arrow indicates spikelet primordium (SP) at the base of elongating spikelet bract primordium (sbp). im- inflorescence meristem, pb- peduncular bract, sbp- spikelet bract primordium, sp- spikelet primordium, vs- vascular strands. The bar represents 100 µm.

Farther along in developmental stage 1C, expansion of the spikelet bracts occurred upward from the base (acropetal). At the base of these lateral growths spikelet primordia were seen at leaf stage F-1 in normal and F-2 in mantled (Figure 7.5). The primordia of these tissues had large nuclei stained blue-black by naphthol blue-black staining solution (Figure 7.3, 7.4, 7.5). This intense and homogenous staining marked the presence of proteins, in this case histones, suggesting a condensed state of chromatin of these cells, owing to the high meristematic activity. The normal and mantled samples showed similar morphology and were still indistinguishable (Figure 7.5).

(1D) The Development of floret primordia: The rachis/main axis of the inflorescence and the spikelets followed a similar pattern of development. Like the spikelet bracts and spikelet primordium developed on the main inflorescence axis, floral bracts and floral primordium developed acropetally on the spikelets. At leaf stage F3 or F0 in normal and mantled respectively, spikelets could be seen bearing floral bract primordia (Figure 7.6 A, B). Floral meristems developed at the axis of floral bracts and were similar in form to that of the rachis and spikelet meristems (Figure 7.6 C, D). This was the earliest stage where the sex of the inflorescence could be distinguished. A male spikelet bears 400-1500 staminate flowers, as opposed to a female spikelet that bears 5-30 floral triads both subtended by floral bracts. Thus, the spikelet morphology, specifically the number of floral bracts per spikelet, could be used as an indication of sex. However, it may be noted that at this stage the same could be distinguished by naked eye observation of the inflorescence morphology.

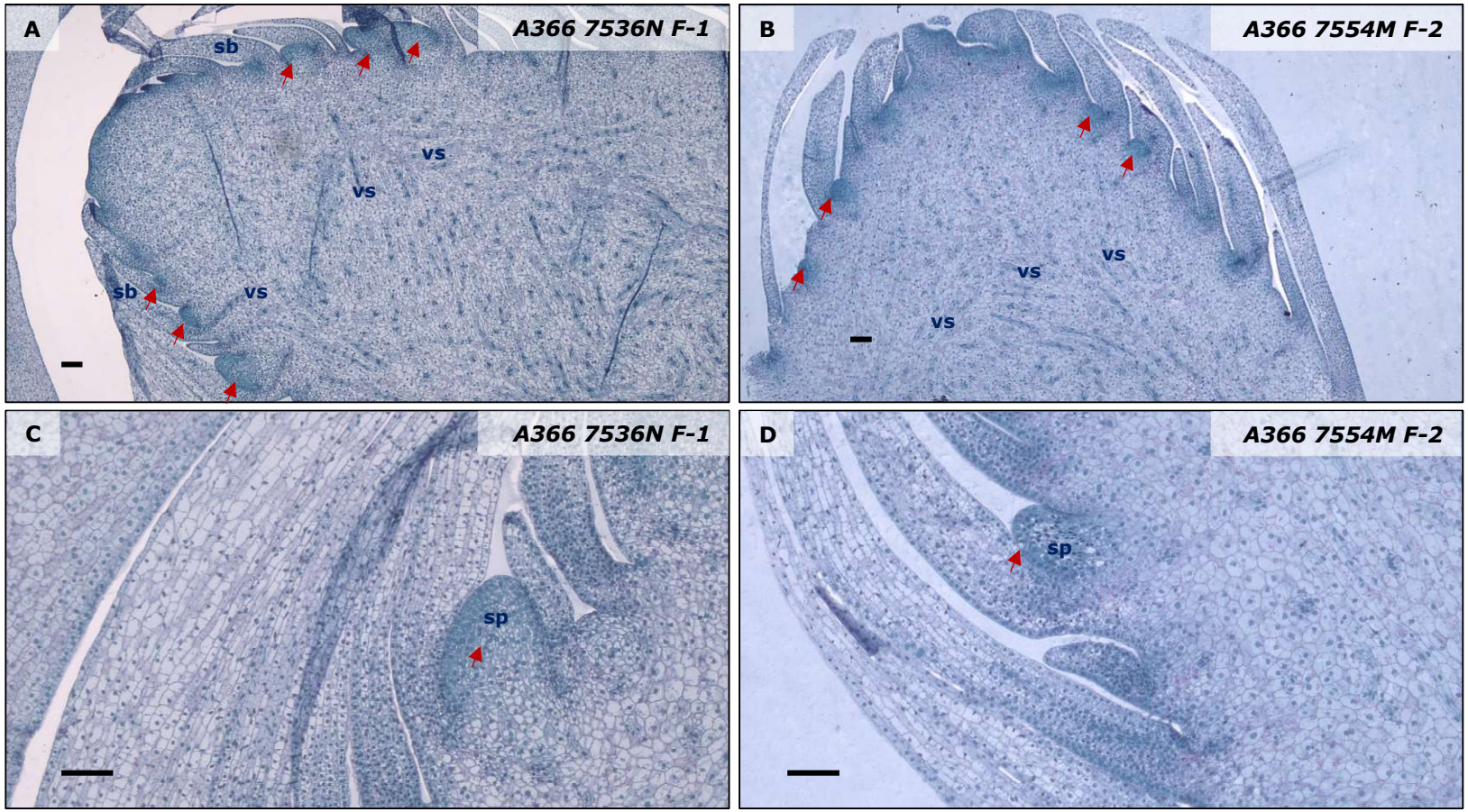

Figure 7.5 Early Inflorescence Development at Developmental Stage 1C. Sections of young inflorescences at leaf stage F-1 in normal (A, C) and F-2 in mantled (B, D) showing development of spikelet primordia (sp). Red arrows indicate spikelet primordia at the base of elongating spikelet bract. sb- spikelet bract, sp- spikelet primordium, vs- vascular strands. The bar represents 100 µm.

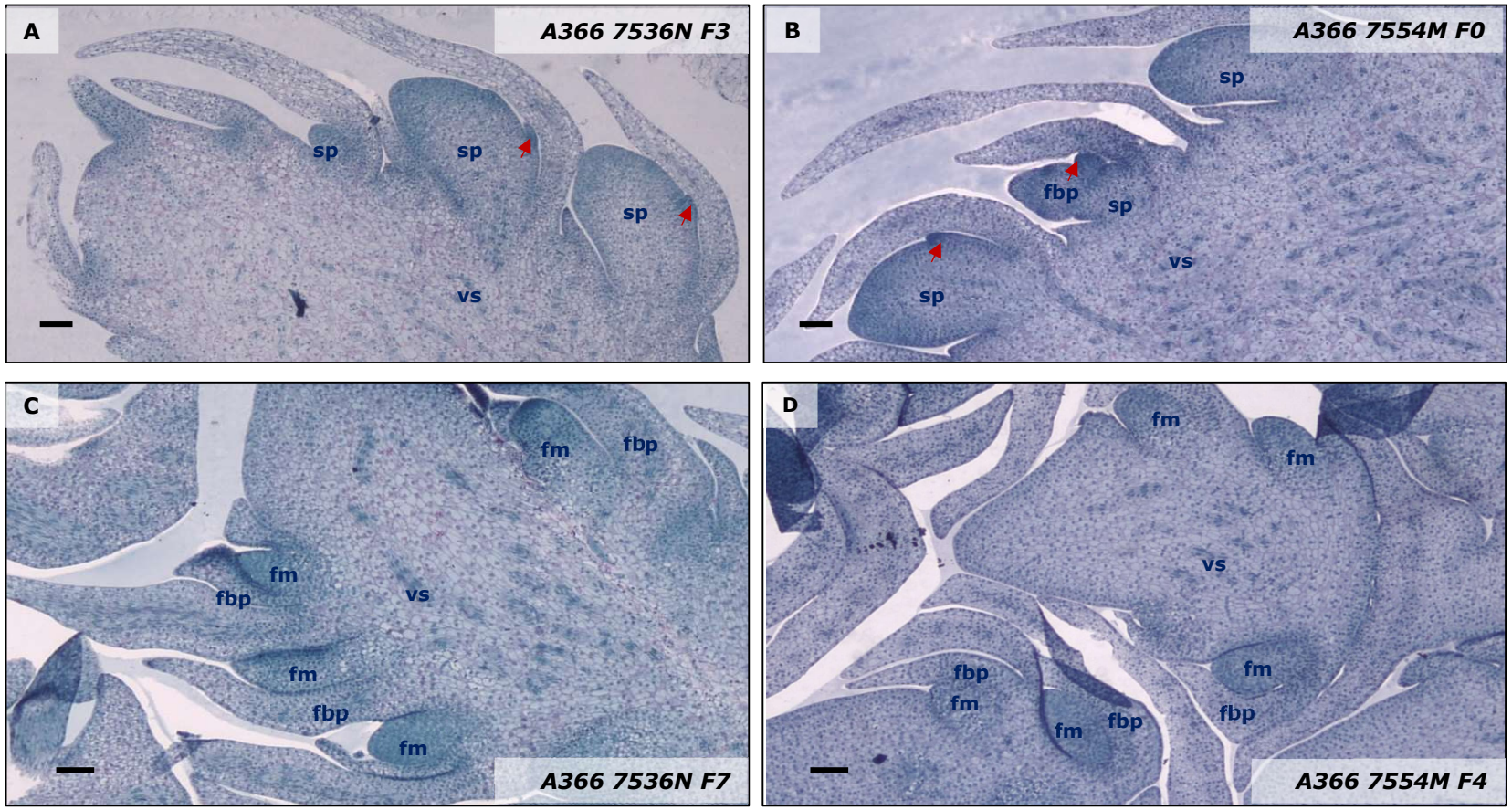

Figure 7.6 Early Inflorescence Development at Developmental Stage 1D. Sections of young inflorescences at leaf stage F3 in normal (A) and F0 in mantled (B) showing development of spikelet primordia and at leaf stage F7 in normal (C) and 4 in mantled (D) showing development of floral bract and floral meristem. Red arrows indicate floral bract primordia (fbp). sp- spikelet primordium, fbp- floral bract primordium, fm-floral meristem, vs- vascular strands. The bar represents 100 µm.

7.3.4. Development of Floral Triads

The floral meristem developed into a sympodial floral triad in a helicoid arrangement. The lateral staminate flowers were formed first followed by the central pistillate flower spirally around the axis of the spikelet. By leaf stage F6 or F7, the spikelets belonging to young inflorescences (YI, predicted stage <1) showed developing floral triads (Figure 7.7) resembling early young triads of predicted stage 1 (YT1, Figure 7.8). The primordia of the individual florets of the triad were distinguishable at this stage; except, the staminate flowers were ahead in development and formed bracteoles and perianth organs first (Figure 7.7). Based on the samples studied, no structural difference could be observed in relation to the difference in age of the palm (young/ mature) or phenotype (mantled/ normal) (Figure 7.7 and 7.8).

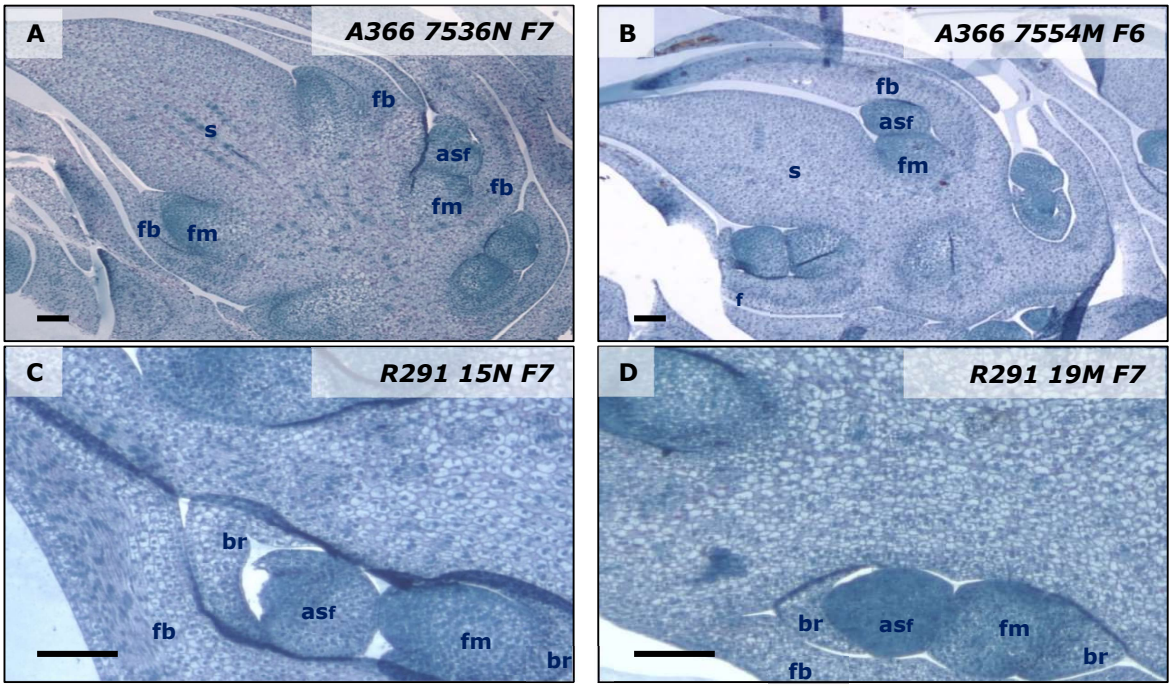

Figure 7.7 Floral Development in Young Inflorescences of Developmental Stage 1D. Section of young inflorescences at leaf stage F7 in young (A) and mature (C) normal palms and at leaf stages F6 and F7 in young (B) and mature (D) mantled palms. Meristems that will develop into the individual flowers of the floral triads are seen subtended by the bracteoles. s- spikelet, fb- floral bract, asf- abortive staminate flower, fm- floral meristem of pistillate flower, br- bracteole. The bar represents 100 µm.

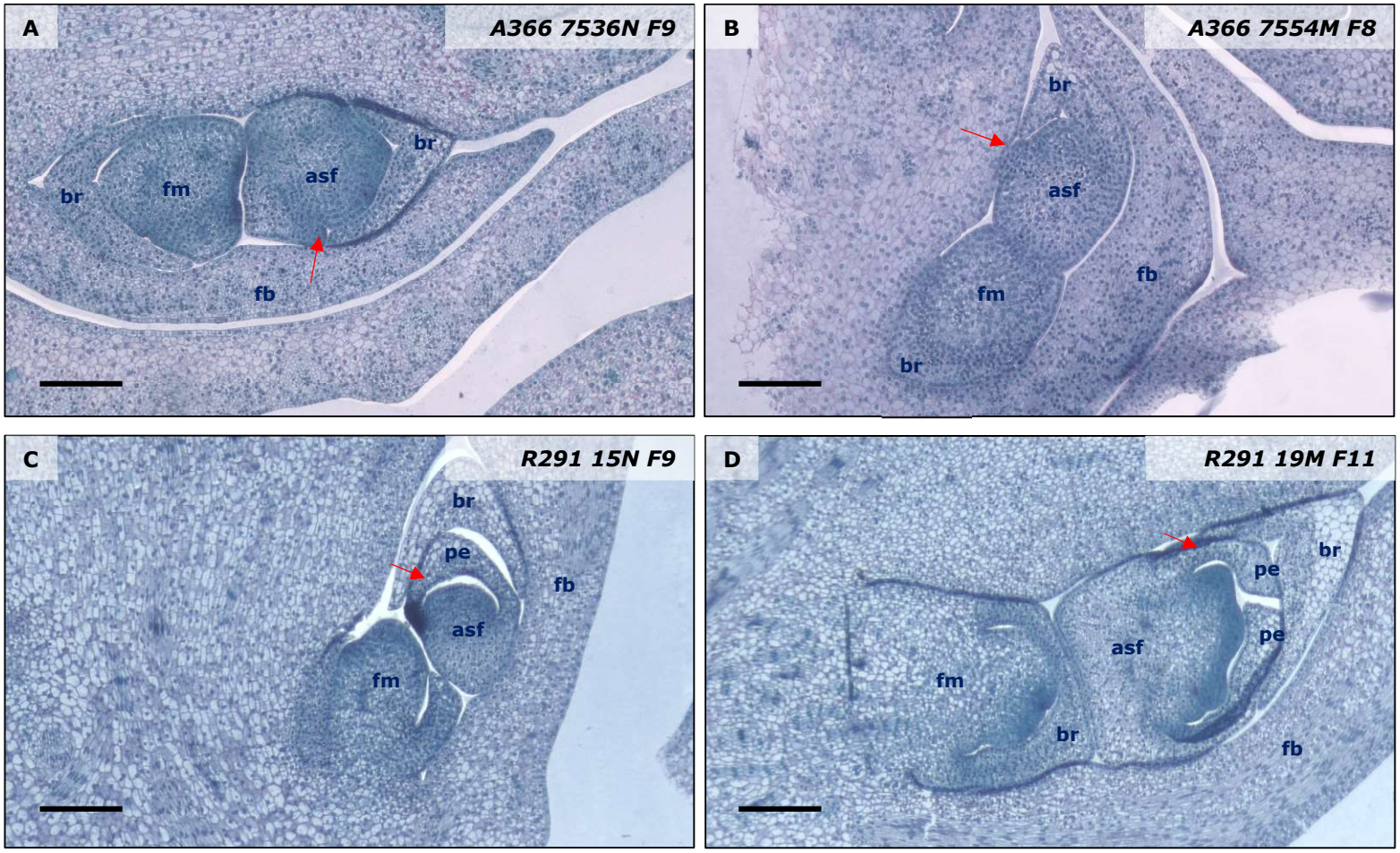

Figure 7.8 Floral Triad Development in Stage 1 Young Triads. Sections of inflorescences at leaf stage F9 in young (A) and mature (C) normal palms and at leaf stages F8 and F11 in young (B) and mature (D) mantled palms. Development of perianth organs (red arrows) is seen in the abortive staminate flowers of the floral triads. Meristems of pistillate flowers of the same triad are slightly behind in development. fb- floral bract, fm- floral meristem of pistillate flower, br- bracteole, pe- perianth. The bar represents 100 µm.

Young Triads 1: At developmental stage Young Triad 1, the meristematic primordia of each of the flowers of the triad formed its own floral bract (bracteole) followed by the perianth (Figure 7.8, 7.9). The perianth in oil palm consists of the calyx (sepals) and corolla (petals), which are morphologically similar and together are referred as tepals (Figure 7.10).

In the case of the mantled samples, histology of floral triad initiation was similar to that in equivalent samples of normal. No observable alterations were visible in the perianth organs or floret meristems at this stage (Figure 7.9).

Young Triad 2: While the abortive staminate flowers had already formed sepals, and petals, the pistillate flower was observed to have only started developing sepals pointing towards the same developmental lag as observed in earlier stages (Figures 7.9, 7.10).

Mantled florets of Young Triad 2 appeared bulkier than their normal complements, but this could have been a difference in the point of development of the compared stages, that is the mantled sample could have been a touch further along in the developmental process and hence bigger (Figure 7.10).

The difference in the size and number of spikelets and florets in young clones compared to mature clones is evident (Figure 7.8). While the developmental process itself follows the same pattern, the young palms will bear smaller inflorescences with a lower number of spikelets and florets (Chapter 4).

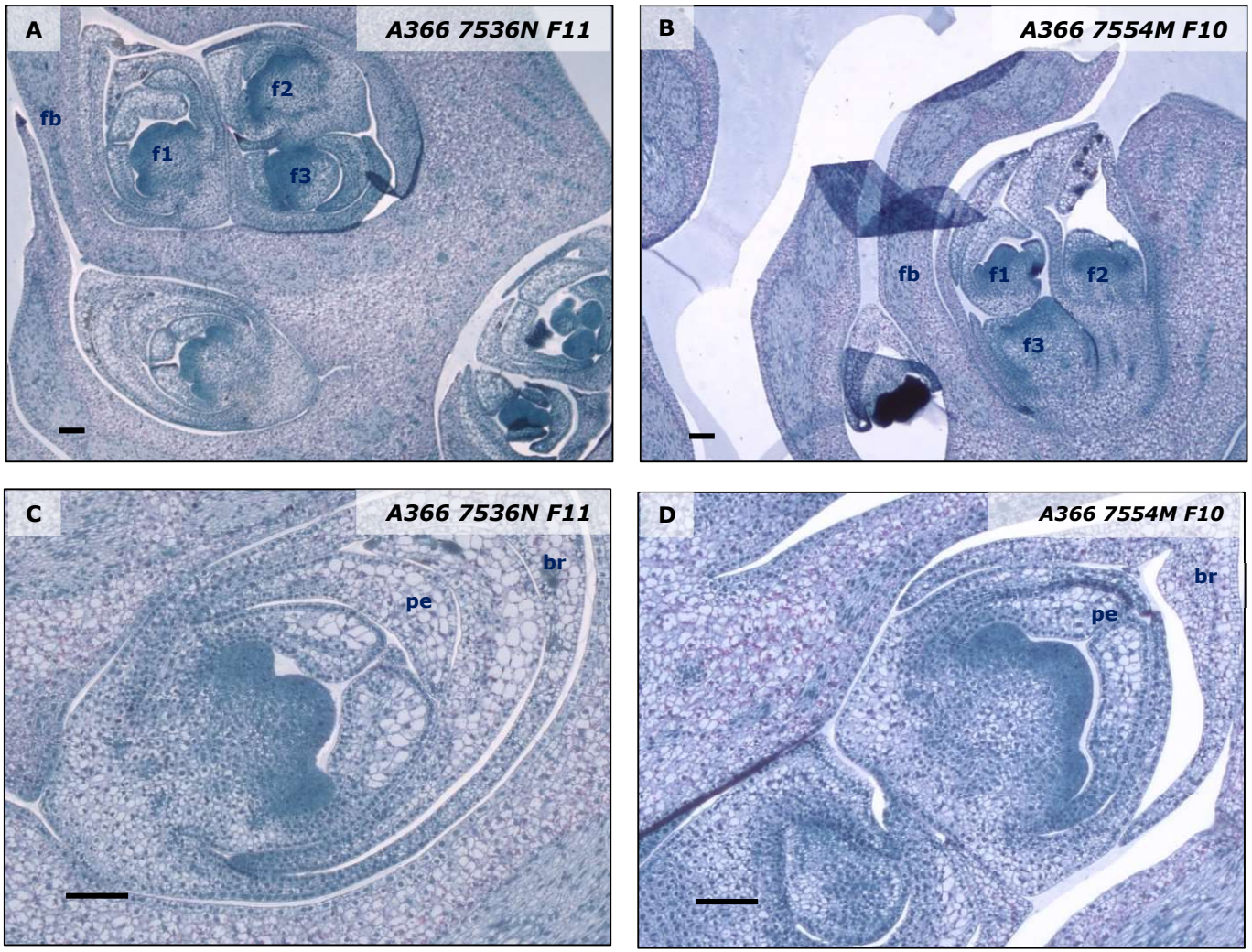

Figure 7.9 Perianth Organ Development in Stage 1 Floral Triads. Sections of inflorescences at leaf stage F11 in a young normal palm (A, C) and at leaf stage F10 in a young mantled palm (B, D). A and B shows floral triads and C and D shows details of perianth organs of individual florets in the triads. fb- floral bract, f1, f2, f3- florets of floral triad, br- bracteole, pe- perianth. The bar represents 100 µm.

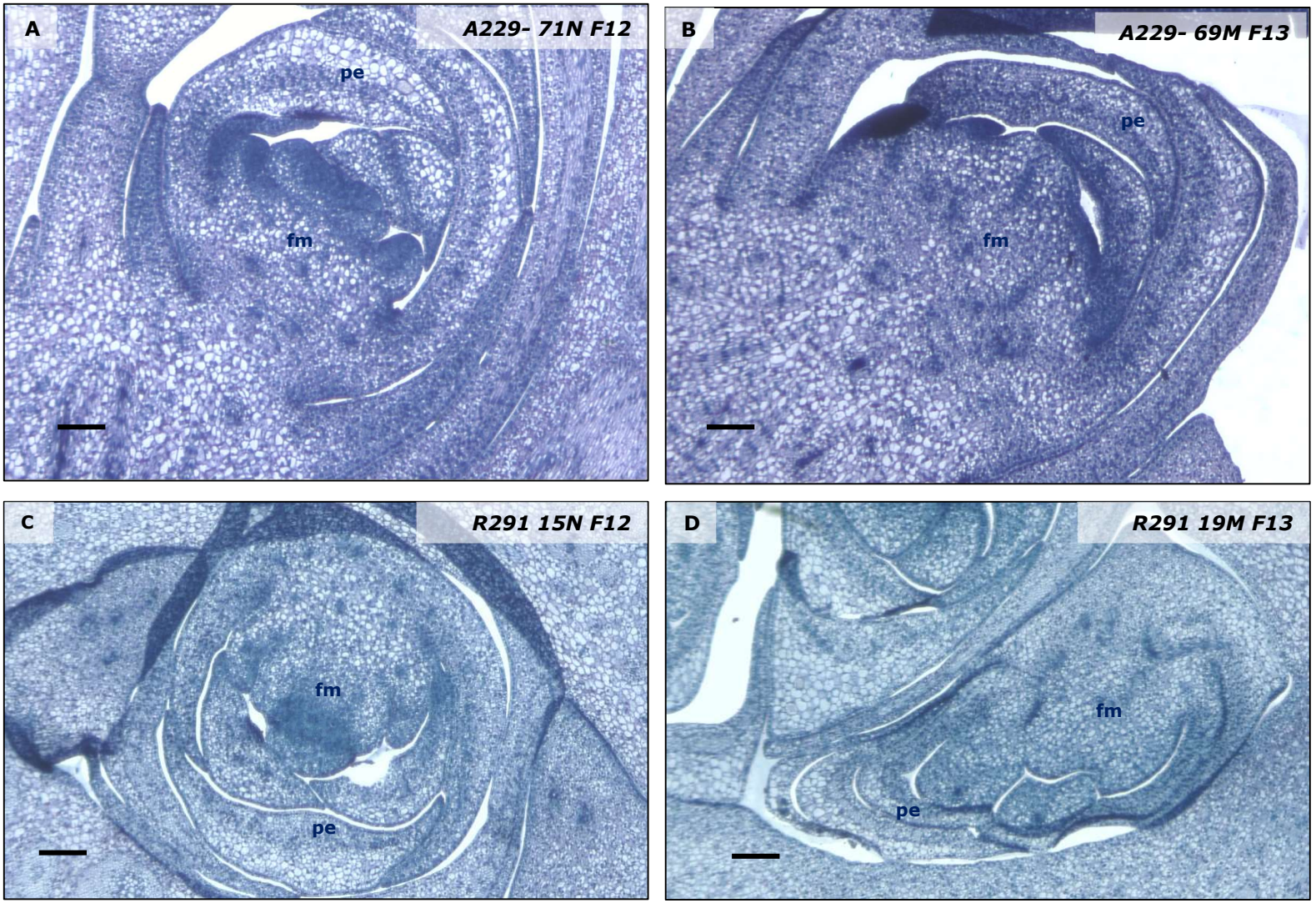

Figure 7.10 Floral development in Stage 2 Young Triads. Sections of inflorescences at leaf stage F12 in normal (A, C) and F13 in mantled (B, D) mature palms of clones A229 and R291. fm- floral meristem, pe- perianth. The bar represents 100 µm.

Floral Triads 3 & 4: Floral meristems of the triad were enclosed by bracteoles, sepals and petals at the developmental stage Floral Triads 3. All three were relatively similar in size initially (Figures 7.9, 7.10) but, eventually, the growth halted in the staminate flowers and the pistillate flower increased in size beyond its companions (Figure 7.11). The stalks of the staminate flowers elongated during development and were relatively positioned above the pistillate flower at this point.

In the pistillate flower, petals showed some amount of polyphenol accumulation. Organogenesis of inner whorls were also in progress, developing carpels were seen in normal flowers and a meristematic cluster where the ovary will soon develop (Figure 7.11). Mantled and normal flowers were distinguishable at this stage since pseudocarpels were visible in the cross-section of mantled pistillate flowers (Figure 7.11). No morphological changes were observed in petals, which also undergo a homeotic transformation to sepal like structures (Jaligot *et al*., 2018).

Interestingly, the abortive staminate flowers of mantled flowers at floral triad stage 3 and 4 showed normal development. That is, they developed anthers with pollen sacs containing a mass of dividing cells, the microsporocytes (Figure 7.12). This was contrary to previous reports. Adam *et al*. (2005) reported homeotic transformation of reproductive whorls of accompanying staminate flowers (or abortive staminate flowers; ASFs) of floral triads in mantled female inflorescences. The observation was made in inflorescences from 100% mantled palms; hence it cannot be due to a lower expression of mantled phenotype. This may be due to a sex specific differential expression of mantled in this clone (See section 7.3.8).

Developing carpels and stamens were distinguishable based on vasculature as well. Carpels had central vascularization whereas stamens of abortive staminate flowers (and functional staminate flowers of male inflorescence in section 7.3.6) had peripheral vascularization (Figures 7.11, 7.12)

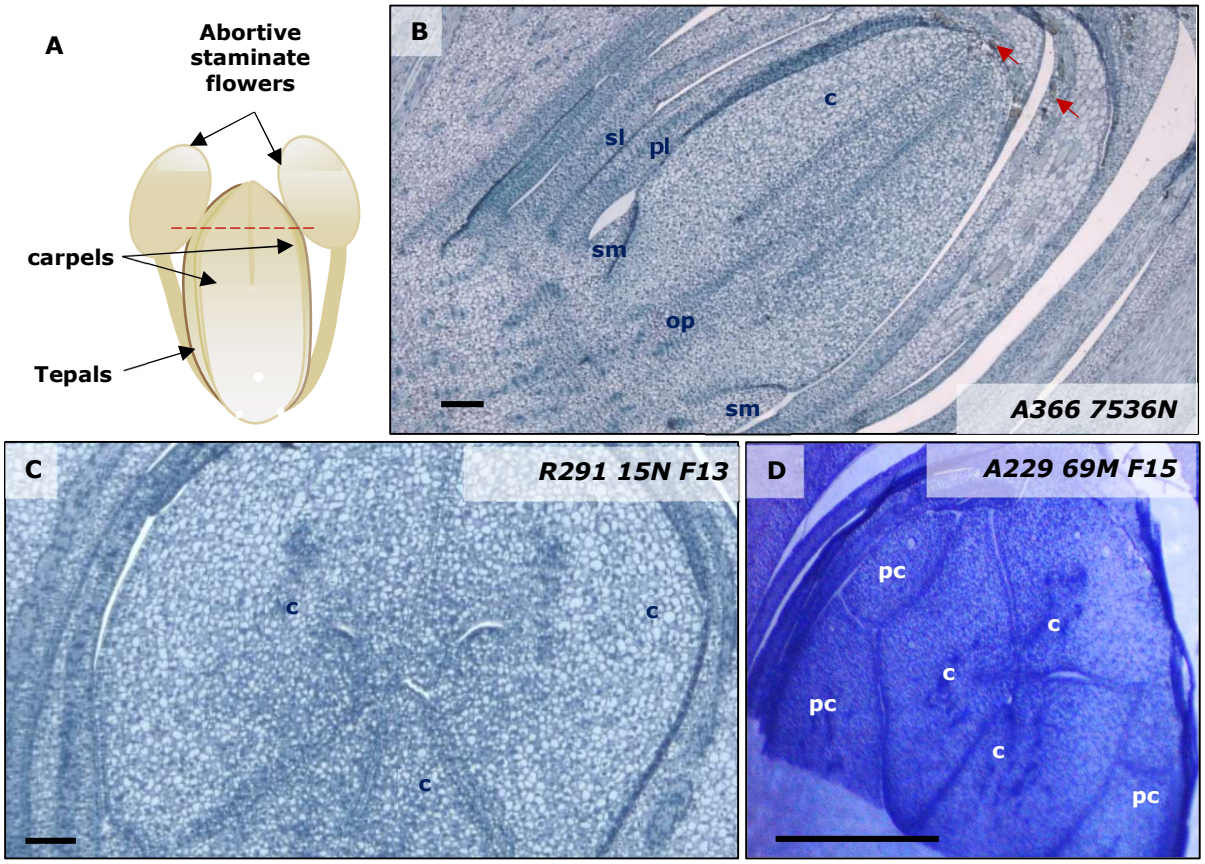

Figure 7.11 Pistillate Flower Development in Stage 3 Floral Triads. A. Diagram showing key features of pistillate flower at developmental stage floral triad 3 (FT3) B. Longitudinal section of normal pistillate flower at leaf stage F15 in young palm belonging to clone A366. C. Cross section of normal pistillate flower at leaf stage F13 of mature palm belonging to clone R291. D. Cross section of mantled pistillate flower in mature palm belonging to clone A229. Red line in A corresponds to rough position of cross sections (C, D). Red arrows show polyphenol accumulation in petals (B). sl- sepal, pl- petal, sm- staminode, op- ovary primordia, c- carpel, pc- pseudocarpel. The bar represents 100 µm.

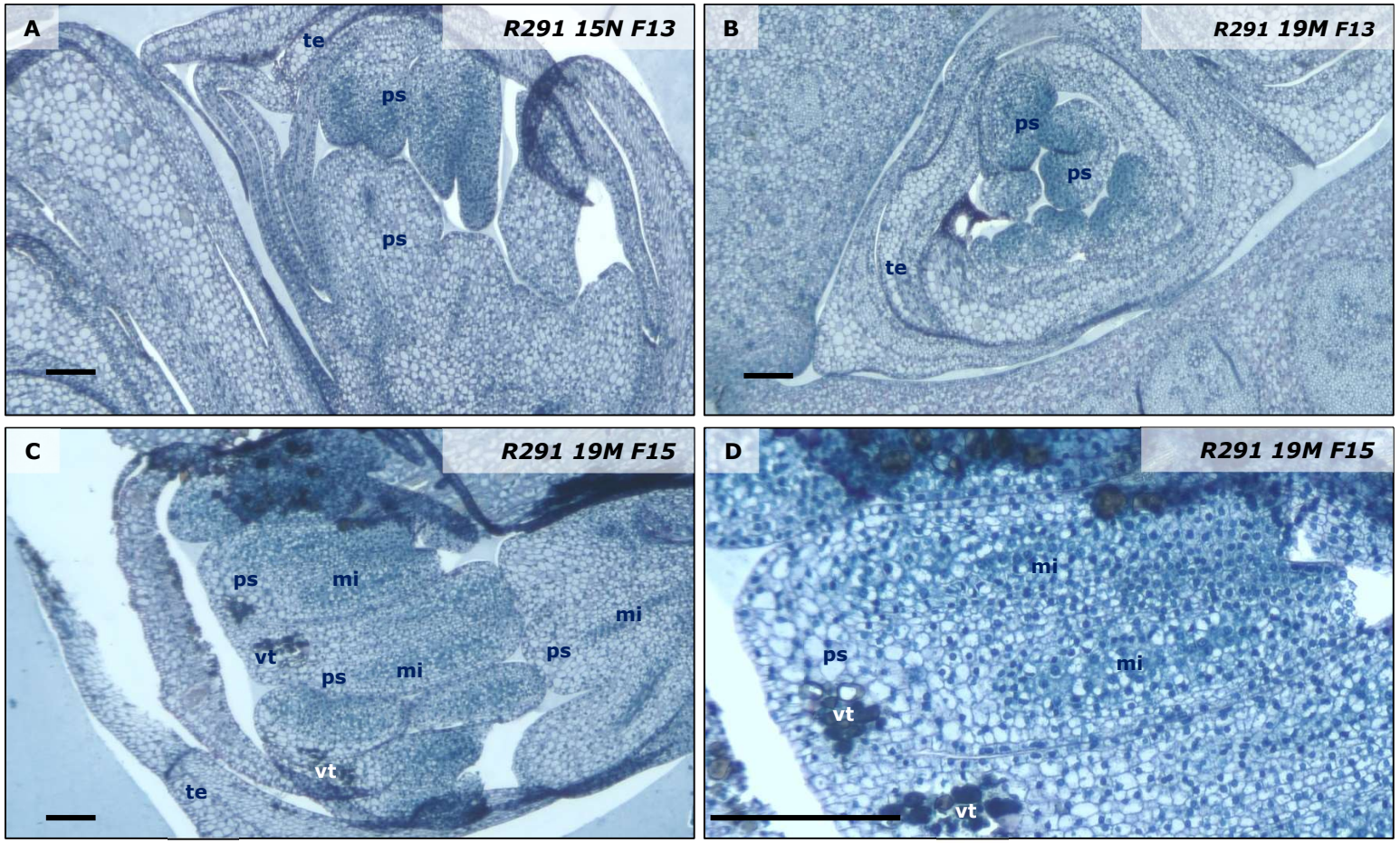

Figure 7.12 Histology of Normal and Mantled Abortive Staminate Flowers. (A) Section of a normal spikelet at floral developmental stage Floral triad stage 3 (FT3). Sections of mantled spikelets at young triad stage 2 (YT2, B) and floral triad stage 4 (FT4, C, D). te- tepals, mi- microsporocyte, ps- pollen sac, vt- vascular tissue. The bar represents 100 µm.

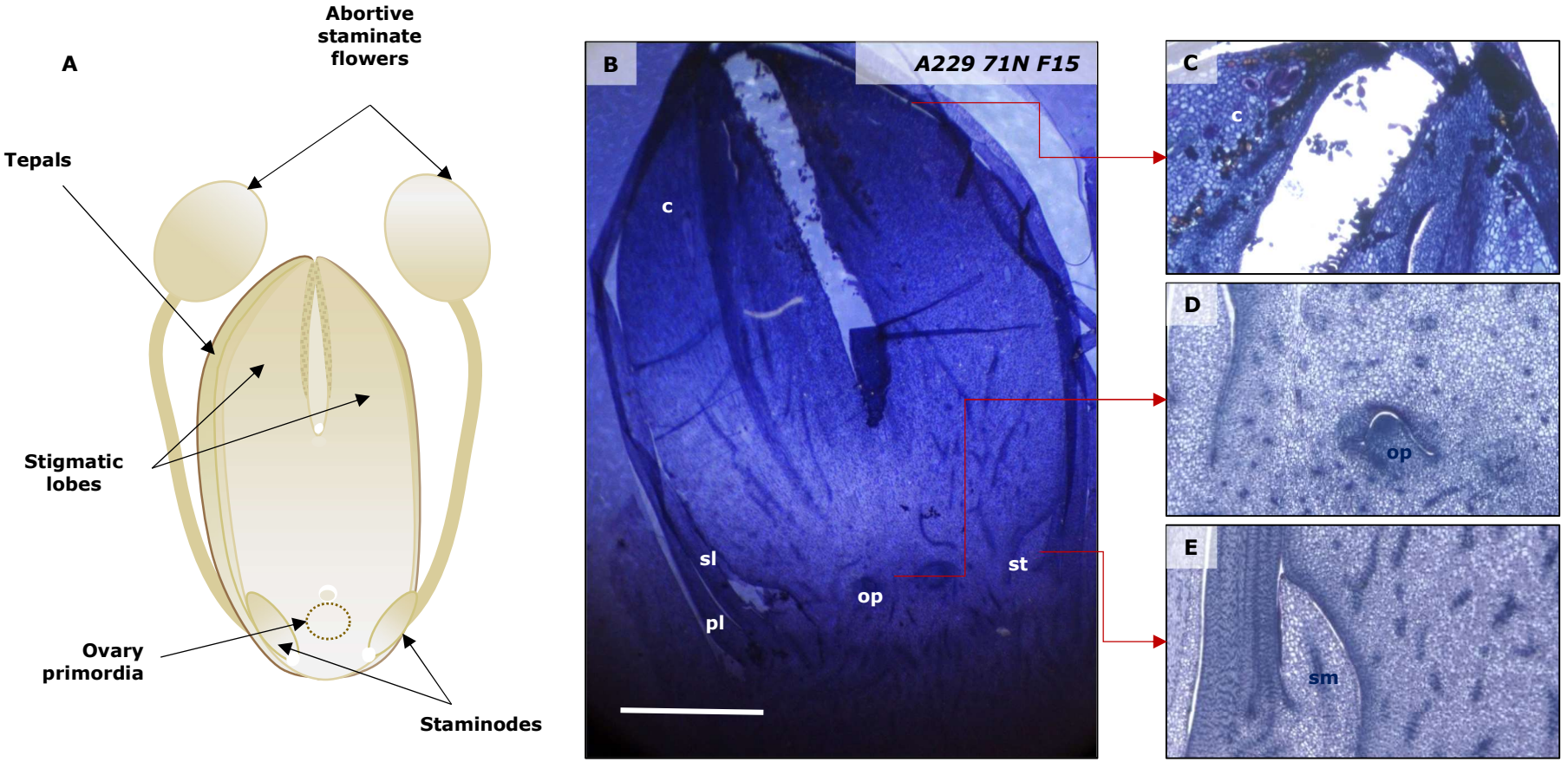

Figure 7.13 Normal Pistillate Flower Development in Stage 4 Floral Triads. (A) Diagram showing key features of normal pistillate flower at developmental stage floral triad 4 (FT4). (B) Longitudinal section (LS) of normal pistillate flower at leaf stage F14 in mature palm belonging to clone A229. Location of features shown by red arrows. (C) LS of stigmatic lobes. (D) LS of ovary primordia. (E) LS of staminodes. sl- sepal, pl- petal, sm- staminode, op- ovary primordia, c- carpel, pc- pseudocarpel. The bar represents 100 µm.

Pistillate flower of Floral Triads 4: At developmental stage 4, the normal pistillate flower showed developing ovary primordia, aborted staminodes, and polyphenol accumulation on the distal end of stigmas (Figure 7.13). Pseudocarpels were seen in the case of mantled flowers, and they were indistinguishable from carpels in histology. The epidermis of pseudocarpels showed anticlinal divisions and the center of the pseudocarpels were highly vascularised. (Figure 7.14).

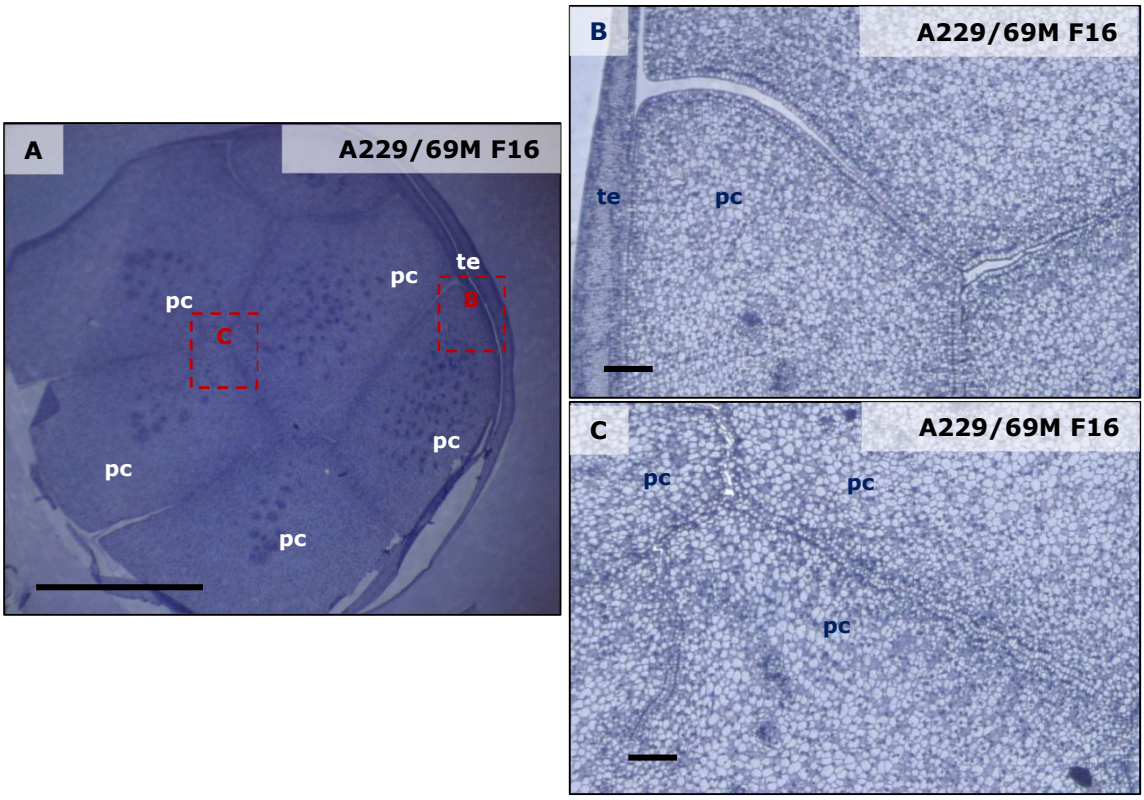

Figure 7.14 Mantled Pistillate Flower Development in Stage 4 Floral Triads. (A) Cross section of pseudocarpels of mantled pistillate flower. (B, C) Higher magnification of features of pseudocarpels, locations indicated in A with red dotted boxes. te- tepals, pc- pseudocarpel. The bar represents 100 µm

7.3.5. Maturation of Pistillate Flower

Beyond predicted stage 4, the pistillate flower develops an ovary (Figure 7.15), but the accompanying staminate flowers never develop pollen, as the microsporocytes degenerate. Before anthesis of the pistillate flower, the abortive staminate flowers become necrotic and are shed/degraded. Unfortunately, none of the microscopic sections showed clear anatomy of the ASFs at this stage.

The pistillate flower showed staminodes at the base, which were similar in size as seen at the previous stage but with polyphenol accumulation in the distal end. The cells were highly vacuolated like permanent tissue (as opposed to meristematic tissue). Carpels were joined at the base but at the upper portion of the stylar canal there were three deep grooves corresponding to the stigmatic lobes. At the distal end on the lateral face, the stigmatic lobes showed vacuolar polyphenol accumulation and an extracellular matrix formed by secretions. The stigmatic lobe and the style were heavily vascularised in both normal and mantled samples. However, in mantled samples, pseudocarpels were seen in the place of staminodes (Figure 7.15).

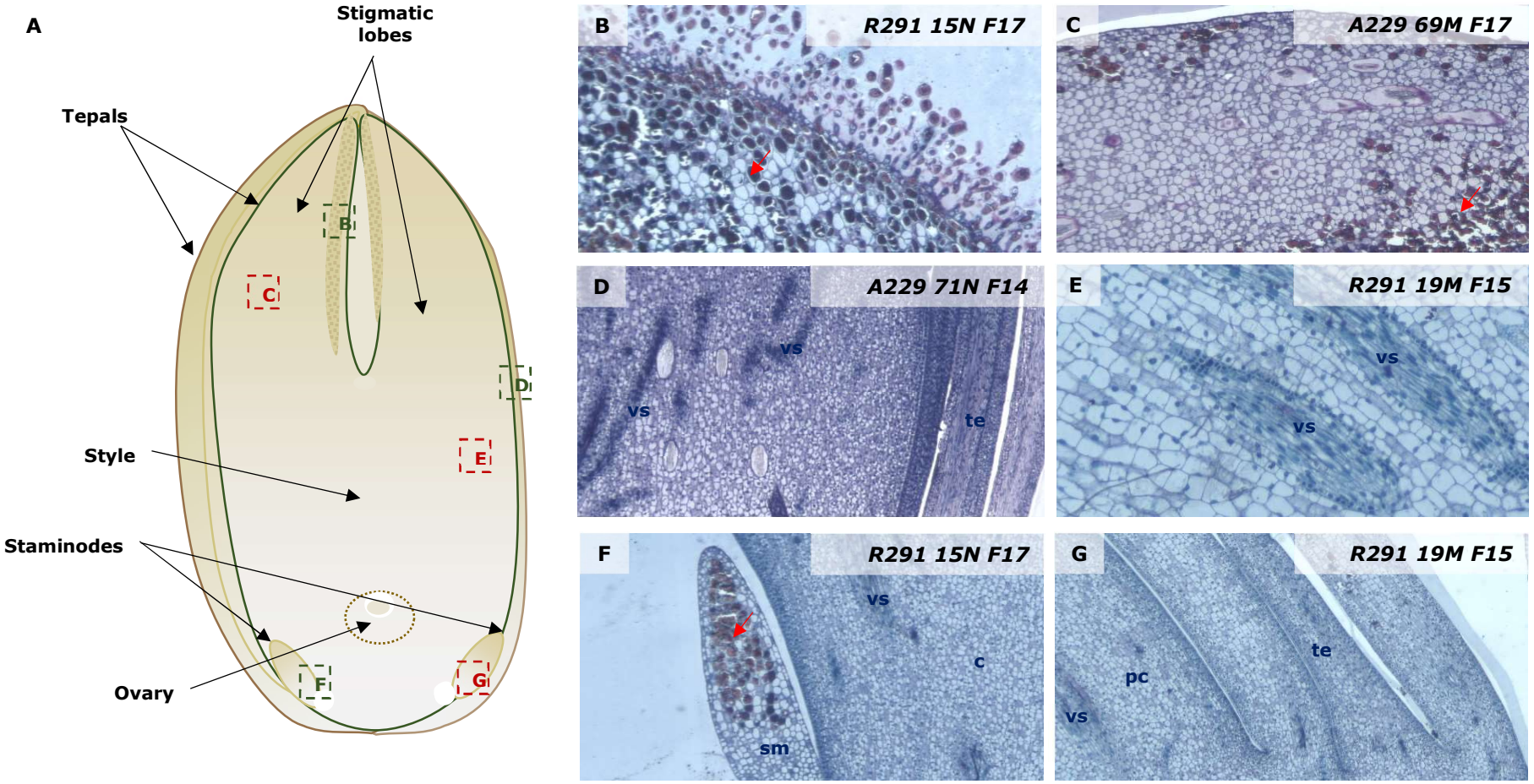

Figure 7.15 Pistillate Flower Development beyond Predicted Stage 4. A. Diagram showing key features of normal pistillate flower past predicted stage 4. Location of microscopic sections is shown in the diagram by green boxes for normal (B, D, F)and red boxes for mantled (C, E, G). B, C. Magnified view of stigmatic lobe showing vacuolar polyphenol accumulation in normal (B) and mantled (C) pistillate flowers. D, E. LS of carpel showing vascularization in normal (D) and mantled (E) pistillate flowers. F, G. Magnified view of base of the flower in normal (F) showing presence of staminode and in mantled (G) staminodes are absent. te-tepal, sm- staminode, c- carpel, pc- pseudocarpel, vs- vascular strip, red arrows indicate polyphenol accumulation.

Mature Flower: At predicted stage >4, within the developmental category Mature Flower/ Inflorescence (MF), florets were separated from spikelets, cut in half/quarter and fixed for microscopic analysis. The tissue was still too big and hard for sectioning. Microscopic observations (Figure 7.16) showed the maturation of reproductive whorls prior to anthesis in the normal pistillate flower.

Within the tepals at the base of the flowers abortive staminodes (the sterile androecium of female flowers arrested in development) were visible. They showed accumulation of polyphenols, as indicated by the differential staining, at the distal end (Figure 7.16).

The carpels were now fully developed. There were three stigmatic lobes which showed accumulation of polyphenols in their lateral faces. The stylar canal, through which after pollination the pollen tubes will grow, could be seen in the lateral section. At the bottom of the carpels in the center of the florets was a developing ovary. Developing/meristematic tissue could be distinguished by their large nucleus and higher nucleus to cytoplasm ratio. Towards the end of this stage, orthotropous ovules were visible.

In the mantled flowers, highly vascularised pseudocarpels were visible in the place of staminodes. The pseudocarpels were as big as carpels and of identical morphology. The stigmas showed accumulation of polyphenols but to a lesser extent. Mantled pistillate flowers may or may not develop a functional ovary, depending on the severity of the abnormality. Microscopic sections of pistillate flowers in mature mantled palm A229/71 (100% mantled) did not show any developing ovules (Figure 7.16).

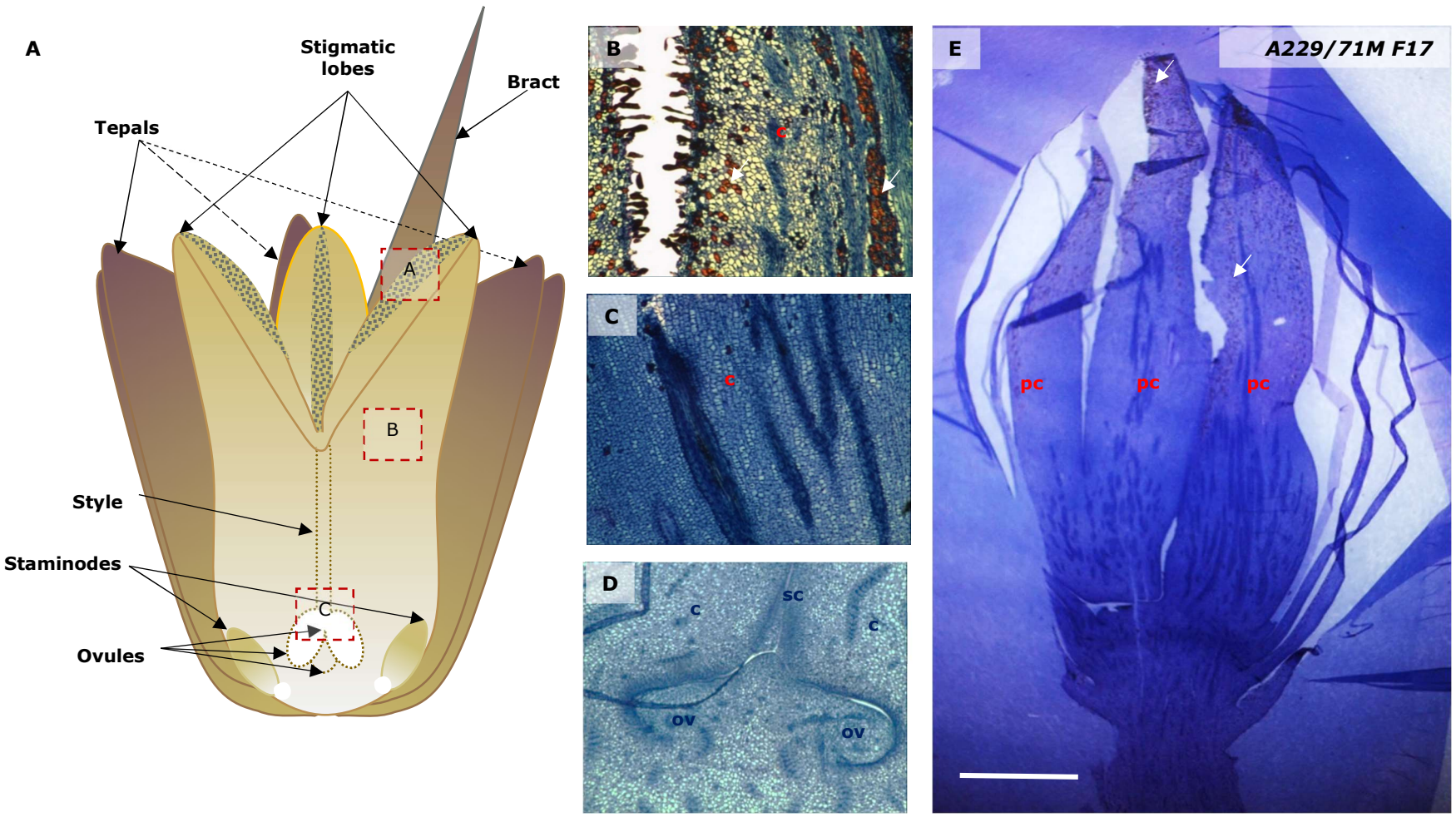

Figure 7.16 Pistillate Flower at Maturity (Developmental Stage MF). A. Diagram showing key features of normal pistillate flower at maturity. Location of features shown by red boxes B. Magnified view of stigmatic lob showing vacuolar polyphenol accumulation. C. Longitudinal section of carpel showing vascularization. D. LS showing two orthotropous ovules at the base of stylar canal. E. Longitudinal section of a mantled pistillate flower at maturity. sc- stylar canal, te-tepal, ov- ovule, c- carpel, pc- pseudocarpel, vs- vascular strip, white arrows indicate polyphenol accumulation. The bar represents 100 µm.

7.3.6. Development of Staminate Flower

In oil palm, the same palm tree yields both male and female inflorescence alternatingly. In cultivated oil palm, the length of the male phase is short, and hence a low number of male samples were encountered during sampling. Further, most of the samples obtained were from normal palms and mantled palms were found to enter male phase less frequently in comparison (See chapter 9). Besides, the normal and mantled palms sampled together did not enter male phase together hence there were no equivalent male samples available for analysis. As a result, it was not possible to do a comparative analysis between male inflorescences from normal and mantled phenotypes.

Histological analysis of "mantled male samples" were not possible due to two additional reasons. One, the older male inflorescences from mantled palms encountered in the sampling range had already undergone anthesis and were necrotic, thus were unfit for histology. Two, the younger male inflorescences sampled from mantled palms were at developmental stages prior to organogenesis in reproductive whorls, hence homeotic transformation could not be evaluated.

Nonetheless, male reproductive development was explored through histological analysis of normal male samples within the sampling range as presented here. The observations are based on 3 samples at predicted stage 2 and >4. Further analysis of field sampling data in relation to the effect of mantled phenotype on incidence of male phase is discussed in chapter 9, and analysis of oil palm pollen samples from normal and mantled palms of 3 different clones, is included in chapter 9.

Early inflorescence development (Developmental stage 1A and earlier) is identical for male and female inflorescences. Morphological differentiation of sex occurs at spikelet bract initiation at developmental stage 1B. However, identification of sex was possible only at 1D where the development of floret primordia clearly indicated differential morphology of spikelets. Male inflorescence development had fewer visual cues for visual staging, compared to the female, hence the predictive algorithm was used exclusively for classification of developmental stages (chapter 6).

Developmental Stage 2: Histological analysis of developmental stage 2 revealed floral meristem development in spikelets (Figure 7.17). The anatomy of the male spikelets was different from the female. Cylindrical spikelets showed a large number of spirally arranged floral bracts subtending the developing floral meristem. Instead of floral triads, a single staminate flower developed inside the individual floral bracts. The floral meristem itself was similar in morphology but smaller in size compared to that observed in pistillate flowers (Figure 7.6, 7.17).

Development of perianth organs was visible on either side of the meristematic dome enveloping the growing floral meristem. The middle of the spikelet was highly vascularised (Figure 7.17B).

Developmental Stage 4 and Beyond: Rapid development of the pollen sac and microspores was seen in successive leaf stages in developmental stage 4. At leaf stage F14 of palm R291/15N developing pollen sacs were clearly visible (Figure 7.19). The normal staminate flowers carried six stamens. Individual stamens consisted of bilobed anthers and connate filaments (Figure 7.18). Within the anthers, four pollen sacs are formed on

either side of the filament. Polyphenol accumulation was seen in the vascular tissue surrounding the pollen sacs (Figure 7.19 B).

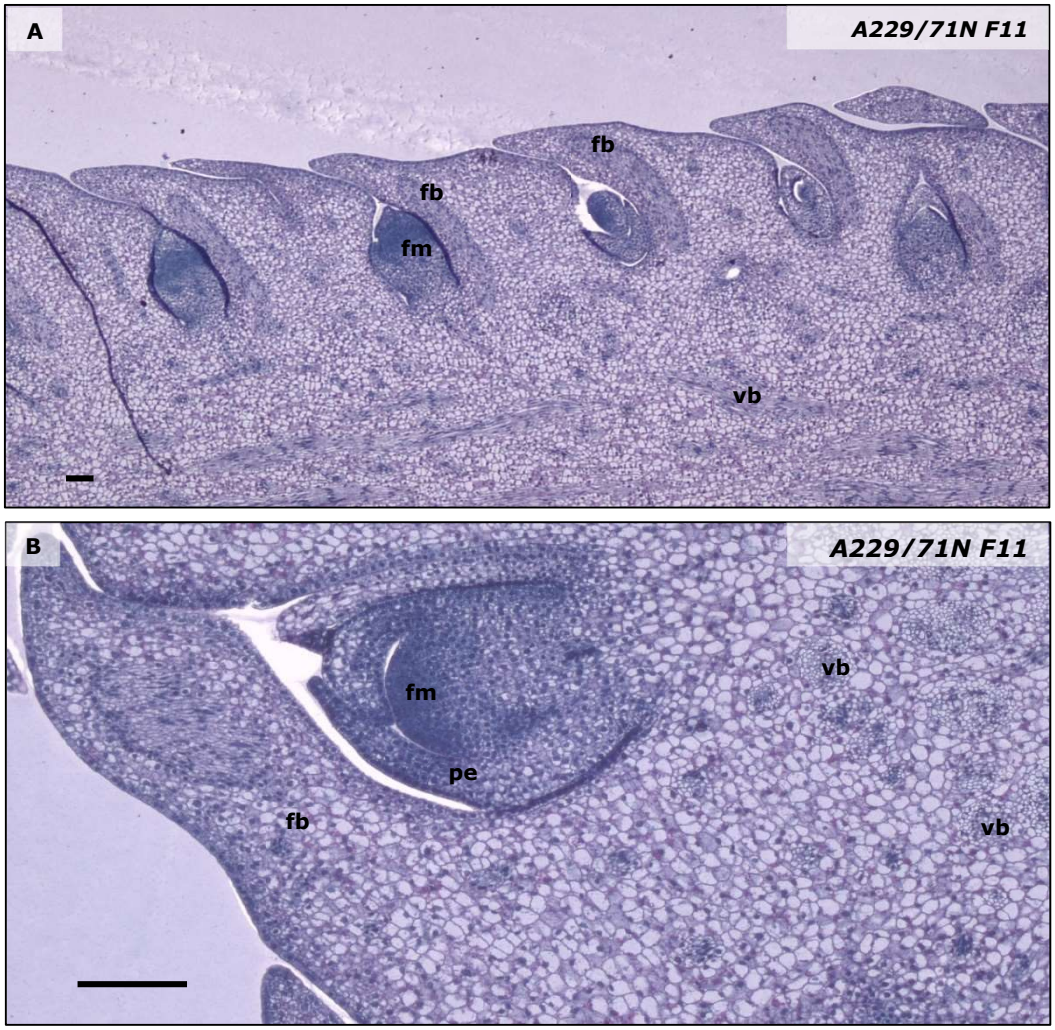

Figure 7.17 Male Inflorescence at Developmental Stage 2. Longitudinal(A) and cross(B) sections of spikelets of male inflorescences at predicted stage 2**.** fb-floral bract, fm-floral meristem, pe-perianth, vs- vascular bundle. The bar represents 100µm.

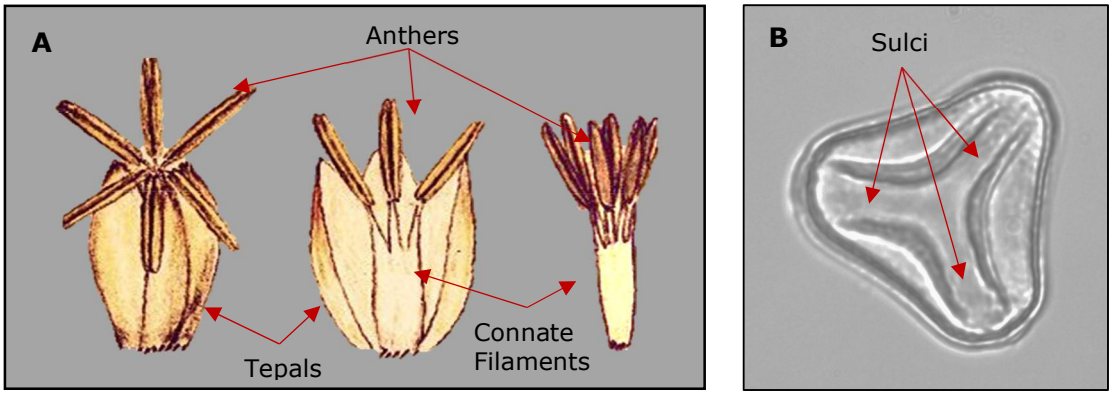

Figure 7.18 Staminate Flowers and Pollen Grains of Oil palm A. Illustration of an individual staminate flower, internal structures, and the androecium (Original Illustration). B. Microscopic image of an oil palm pollen grain. The pollen grain is triangular with rounded angles and shows three distal sulci.

At leaf stage F15 of the same palm (R291/15N) microspores could be seen in the form of tetrads inside the pollen sacs (Figure 7.20). This indicates the completion of the second meiotic division. Tapetum is degenerating at this stage (Figure 7.20 B). Subsequently, at leaf stage F16 (palm R291/15N) free microspores were seen in the pollen sacs (Figure 7.21).

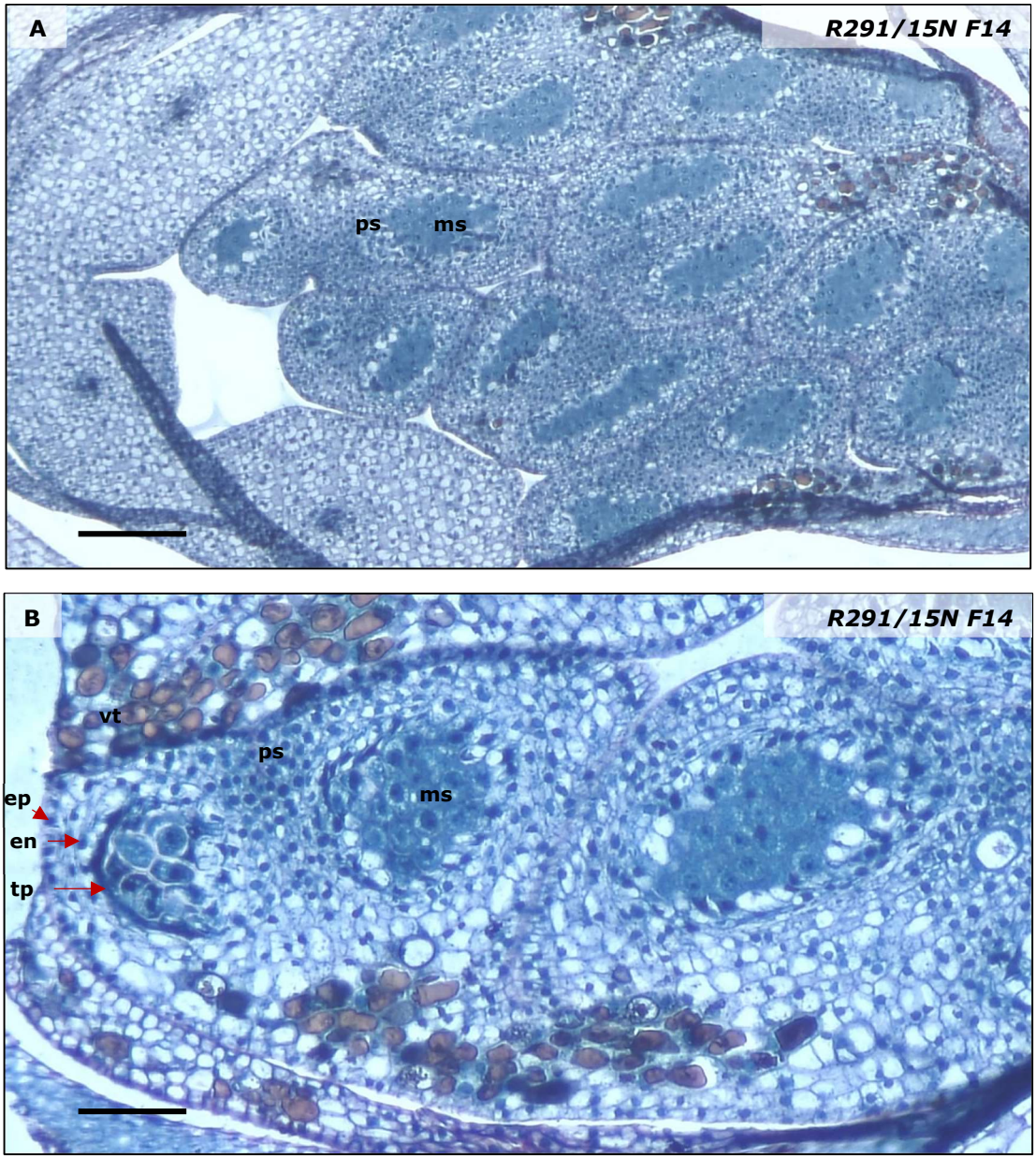

Figure 7.19 Pollen Sac Development at Developmental Stage 4. Cross sections of staminate floret from male inflorescence at leaf stage F14. Images are of magnifications 10x (A) and 100x (B) magnification showing pollen sacs and developing microspores. ps-pollen sac, ms- microspore, ep- epidermis, en- endothecium, tp- tapetum vt- vascular tissue. The bar represents 100um in (A) and bar represents 10um in (B)

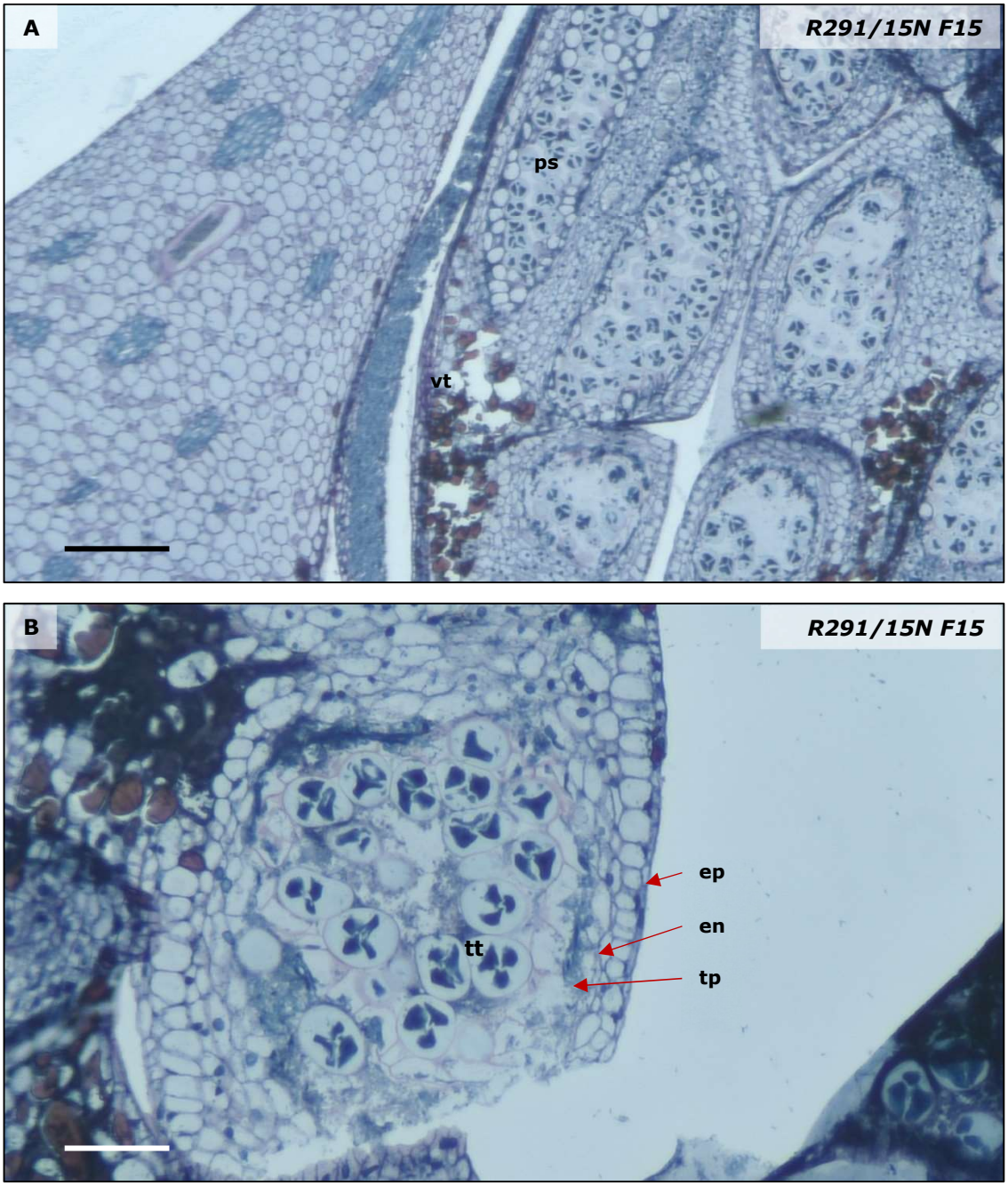

Figure 7.20 Pollen Tetrads of Staminate Flowers beyond Predicted Stage 4. Cross sections of staminate flower from male inflorescence at leaf stage F15, developmental stage >4. Images are of magnifications 10x (A) and 100x (B) showing microspore tetrads. ps- pollen sac, vt- vascular tissue, ep- epidermis, en- endothecium, tp- tapetum, tt- tetrad. The bar represents 100um in (A) and bar represents 10um in (B)

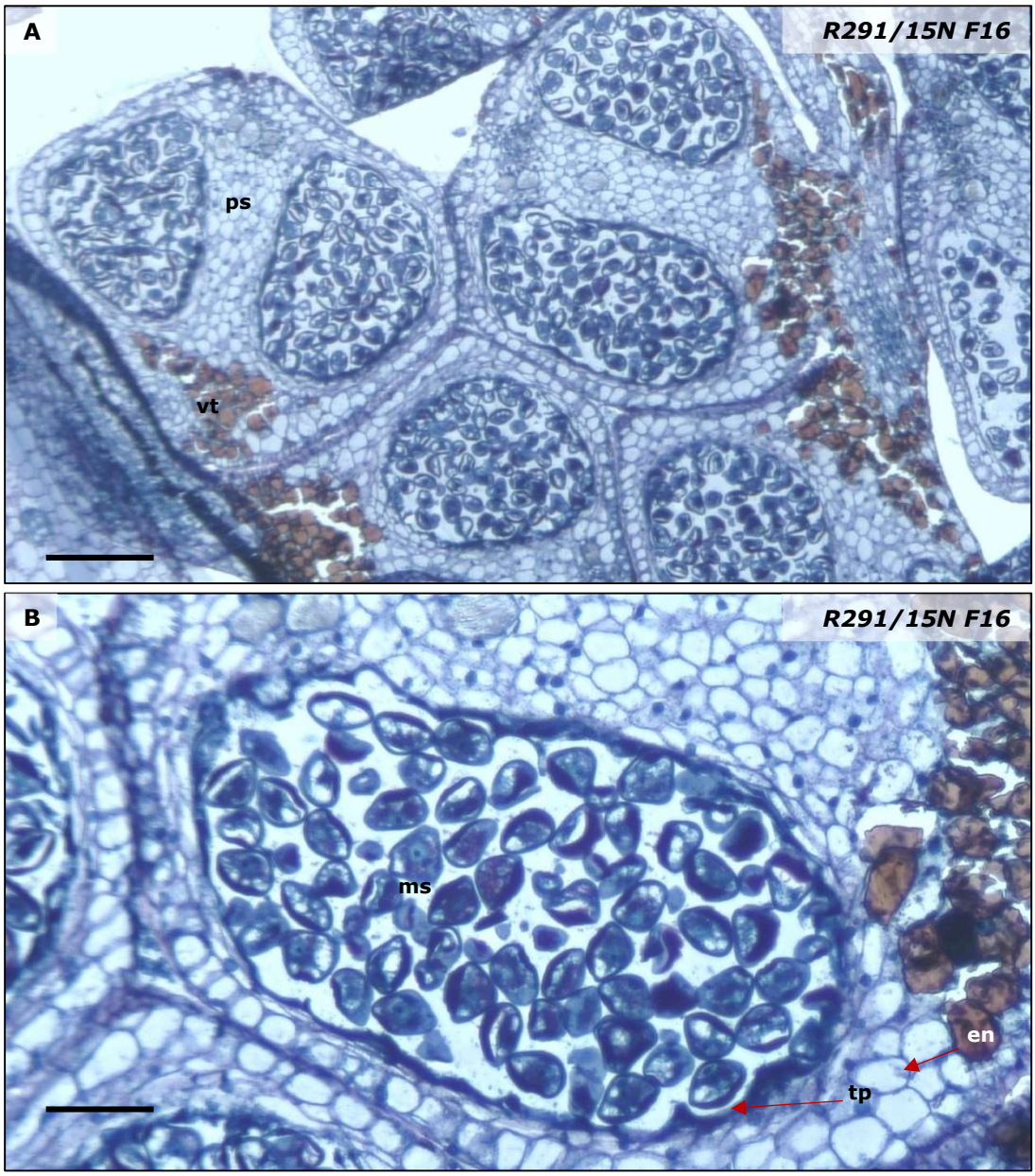

Figure 7.21 Tapetum Degeneration beyond Predicted Stage 4. Cross sections of staminate flower from male inflorescence at leaf stage F16, developmental stage **>4**. Images are of magnifications 10x (A) and 100x (B) showing free microspores in the pollen sacs and degenerating tapetum. ps- pollen sac, vt- vascular tissue, en- endothecium, tp- tapetum, ms- microspore. The bar represents 100um in (A) and bar represents 10um in (B)

Observations from the male samples are summarized in the limited reproductive developmental series in Figure 7.22.

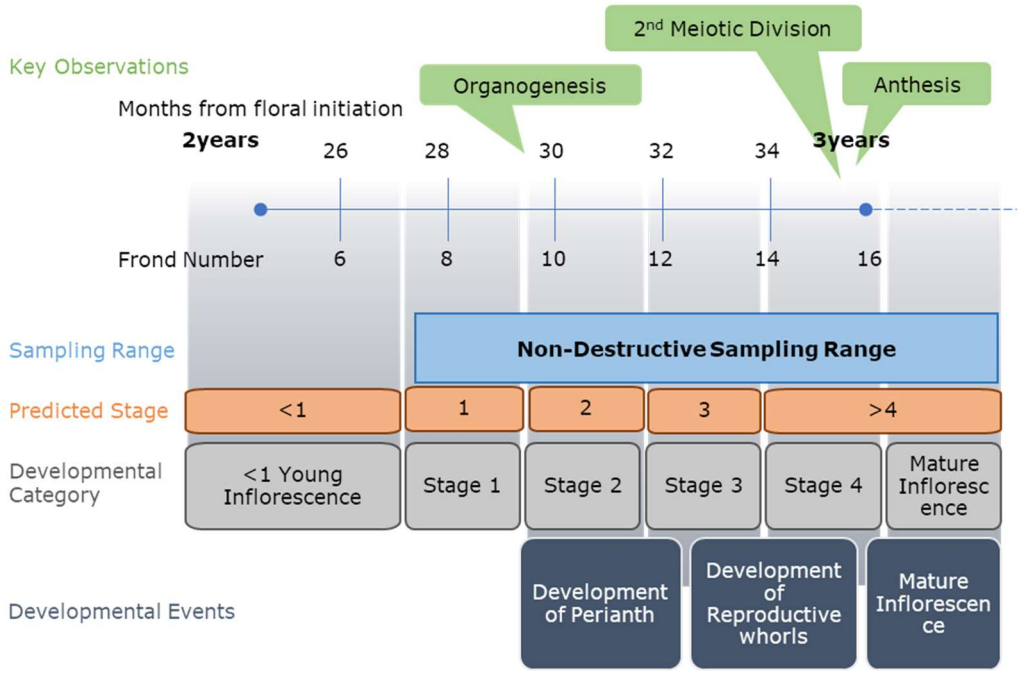

Figure 7.22 Chronological Depiction of Developmental Events in Male Reproductive Development in Oil Palm. Estimated number of months from floral initiation and leaf stage as represented by frond number are depicted in the timeline. Developmental stages 1 to 4 were attainable through non-destructive sampling of sampling range F7 to F18. Organogenesis commenced in functional staminate flowers at developmental stage 2. Development of perianth organs was followed by development of stamens. At Developmental stage 4 developing microspores were visible in the pollen sacs. The second meiotic division was found to occur beyond developmental stage 4, roughly before leaf stage F16. Anthesis occurred earlier than in female inflorescences at approximately F16.

7.3.7. Hermaphrodite Inflorescences

Hermaphrodite inflorescences may be an interesting source of information regarding the effect of mantled phenotype on sex (further explored in Chapter 8). In one hermaphrodite sample that was collected from a ten-year-old mantled palm the male part appeared normal whereas the female florets were visibly mantled. This has previously been reported in naturally occurring homeotic mutant of oil palm *diwakkawakka (Hartley, 1988; Adam et al., 2005).* In another instance what looked like fully developed male flowers in the lower spikelets of a mantled female inflorescence were also

collected from one of the young less severely mantled palms. However, in young clonal palms of three to four years of age in the field many such instabilities are common.

7.3.8. Sex-Specific Expression of Mantled

In the current set of samples, the male inflorescences from mantled palms did not show homeotic transformation characteristic of the phenotype. As per previous description (Adam *et al*., 2005; Jaligot *et al*., 2011; Ooi *et al*., 2019) in the case of mantled male inflorescences, the stamens of the staminate flowers are converted to fleshy carpel like structures, and the flowers produce no pollen. In fact, Beulé *et al*., (2011) described the phenotypic alterations of mantled male inflorescence (stamens to carpels) to be "more drastic" than that observed in mantled female inflorescences (staminodes to pseudocarpels). And thus, mantled male inflorescences were used as the source material for identification of genes differentially expressed between normal and mantled phenotypes (Beulé *et al*., 2011).

However, in this sample set mantled homeotic transformation was limited to female inflorescences. Within female inflorescences phenotypic alterations associated with mantled phenotype were seen only in the pistillate flowers. The abortive staminate flowers of floral triads in mantled female inflorescences exhibited normal development (Figure 7.12). All mantled male inflorescences, including male spikelets of hermaphrodite inflorescences obtained in this study had normal physiology (Plate 6.10). It is worth noting that in *Diwakkawakka* palms, a naturally occurring oil palm mutant similar to mantled, staminate flowers are normal while pistillate

flowers have the same phenotype as those of mantled palms. Further analysis was limited by the sample size (See section 7.3.6).

7.3.9. Inter-species Comparison

The reproductive developmental process was observed to be very similar to that reported in American oil palm, *Elaeis oleifera* (de Farias *et al*., 2018) and Coconut palm, *Cocos nucifera* (Perera *et al*., 2010). In all three palms, the peduncular bract and prophyll protects the young inflorescence during its development. Shortly after inflorescence initiation, the prophyll envelops the inflorescence meristem (Figure 7.2). As the development progress, they become fibrous and eventually necrotic. They then break open, exposing a maturing inflorescence before anthesis.

The same zonation consisting of three tissue layers (L1, L2 and L3) is established in the meristems and largely maintained as separate tissues during development of all cases. L1 forms the epidermis and is responsible for protection and gas exchange. The L2 layer acts as a cell reservoir and develops lateral meristems that develop into subepidermal tissue and new organs. The L3 layer generates the vascular system, for long-distance transport of water and metabolites.

The developmental process consists of multiple growth phases of different growth rates in coconut palm and both oil palm species. Almost two-thirds of the entire duration of reproductive development was spent for slow expansion of the inflorescence meristem to form the inflorescence structure wherewith organogenesis and development of the individual flowers take place rapidly over the last few months.

Interestingly the floral triads of coconut originate from a single floral meristem and not three individual floral meristems as in the case of oil palms. This is indicated by the presence of single floral bract in former as opposed to three bracteoles in latter (Figure 7.7, 7.8). Floral characters like this help delimit monophyletic groups within the *Arecaceae* family and thus have high taxonomic value. The organisation of the flower complex in the inflorescence is a defining feature of subfamily *Arecoidae and* the floral triad is considered to represent the ancestral configuration of the *Cocoseae* tribe to which both coconut palm and oil palms belong (Adam *et al*., 2005; Perera *et al*., 2010).

Coconut palm produces mixed-sex inflorescences where staminate and pistillate flowers are separated spatially and temporally. It is thought-provoking to note that in some of the hermaphrodite inflorescences observed during sampling, the male spikelets underwent anthesis earlier than the female spikelets of the same inflorescence, in a similar pattern (Section 7.3.7). The similarities among these palms show conservation of regulatory mechanisms across species.

Inflorescence development as well as individual flower development in American oil palm, is remarkably similar to that observed here for African oil palm. This would seem reasonable since the divergence of the two species was a result of geographical isolation (Barcelos *et al*., 2002). This detailed understanding of oil palm reproductive development and pollen quality alongside that of the American oil palm will be useful in the development and use of interspecific hybrids for future breeding programmes.

An interesting comparison for future studies will be between mantled somaclonal variant and abnormal somaclonal variant of '*Barhee*' cultivar of date palm. This "low fruit setting phenotype' is associated with formation of parthenocarpic fruitlets with three carpels. In severe cases of this abnormality multicarpel flowers and fruitlets have been reported (Attaha and al-Saadi, 2015). Abd-Elhaleem *et al*., (2020) conducted histological examination of the abnormal phenotype. They reported absence of embryo sac development in the ovary of abnormal flowers, this was similar to observations made in the present study in relation to mantled (Figure 7.16). Sterility, possibly due to absence and atrophy of ovule, is associated with severe mantled phenotype as established in chapter 4. Further the pseudocarpels of mantled flowers do not appear to develop an ovary at all (Figures 7.11-7.16).

Abnormal phenotype of *Barhee* date cultivar was also linked to deformity of stigma that contributes to failure of fertilization. Examination of pollen-pistil interaction in normal and mantled flowers was beyond the scope of the current research, however the protocols prescribed in chapters 3, 4 and 5, developmental characterisation described in chapter 6 and 7 and histochemical protocols for pollen functional quality assessment established in chapter 8 will be useful for such an investigation.

7.4. SUMMARY

A reproductive developmental series from early inflorescence development to floral maturity is presented here (Figure 7.1), enabling comparisons between age categories (three-year-old young clone and ten-year-old mature clones), sexes (pistillate/female, staminate/male and hermaphrodite) as well as phenotype (normal and the epigenetic homeotic mutant, mantled). The reproductive developmental process was also compared to that in American oil palm, and Coconut palm and was found to be very similar.

Results indicate a systematic approach as proposed here is effective in identifying and comparing key developmental events. It is also invaluable characterising mantled reproductive development to reveal novel insights. The comparative reproductive developmental series allowed comparison of mantled inflorescences against equivalent normal counterparts across the developmental process for the first time. This provided the following insights. One, inflorescence development in normal and mantled are indistinguishable by histology until organogenesis. Earliest identification of mantled phenotype is possible at developmental stage FT3. Two, in the current sample set, homeotic transformation associated with mantled was sex specific and only occurred in the pistillate flowers. ASFs of mantled female inflorescences showed normal development.

Oil palm inflorescence meristem is borne deep in the crown of the palm approximately two years before anthesis. During early development of young inflorescences (Developmental category 1) the meristem gives rise to two spathes, prophyll and peduncular bract, which envelops the

developing axis (Developmental stage 1A as seen in figures 7.2 and 7.3). Over the next stages the meristem expands into the central rachis and produces spikelet bracts (also known as rachilla bract) acropetally (Developmental stage 1B as seen in figure 7.4). At the base of the spikelet bracts, spikelet meristem is born (Developmental stage 1C as seen in figure 7.5). At this stage male and female inflorescences are distinguishable by spikelet morphology.

The spikelet meristem follows a similar developmental pattern producing floral bracts acropetally (Developmental stage 1D as seen in figure 7.6). Microscopic sections of male and female spikelets at this stage are distinguishable by the number of floral bracts per spikelets which is substantially higher in male inflorescences (Section 7.3.6). The floral meristems in both male and female spikelets are similar when they first develop within the floral bract, but smaller in the former (Figure 7.6, 7.17). In female inflorescences, the floral meristem gives rise to a sympodial cluster consisting of a central pistillate flower and two accompanying abortive staminate flowers (ASFs) (Developmental stages 1D and YT1 as seen in figures 7.7 and 7.8).

Organogenesis in oil palm flowers, a key stage for developmental studies, is fast and is marked by an exponential increase in the length of the inflorescence at developmental stage YT1. The difference in the size and number of spikelets and florets in young clones compared to mature clones is evident now itself (Figure 7.7, 7.8), inflorescences from young clones are smaller in size. The abortive staminate flowers develop faster than the pistillate flower in the same triad (Development of young triads as seen in

figure 7.9 and 7.10). The ASFs develop perianth organs- bracteole, sepals and petals followed by anthers and pollen sac, but the microsporocytes degenerate without producing functional pollen. Pistillate flowers develop perianth organs and carpels.

No differences were observed in the micro-morphology of developing inflorescences from normal and mantled palms until organogenesis (Sections 7.3.3, 7.3.4). One of the objectives of the analysis was to pinpoint the earliest developmental stage where mantled abnormality can be recognised. Floral triad stage 3 was the earliest mantled inflorescence could be distinguished from their normal counterpart (Developmental stage FT3 as seen in figure 7.11). The differentiation was based on the development of pseudocarpels instead of staminodes, in mantled. Until this point the developing inflorescences were indistinguishable. No morphological changes were observed in the inner perianth, petals, which also undergoes a homeotic transformation at a molecular level. This was in line with previous reports (Jaligot *et al*., 2018).

Unlike previously published data (Adam *et al*., 2005), in the present set of samples, the ASFs showed normal development while the pistillate flower of the same triad was mantled (Section 7.3.4). That is, in the present sample set, the homeotic transformation associated with mantled abnormality (formation of carpelloid structures in the third whorl, androecium) was limited to pistillate flowers, like in the case of naturally occurring mutants of oil palm *Diwakkawakka*. The sex specific expression of mantled also warrants further investigation.

Beyond predicted stage 4, the normal pistillate flower developed an ovary, while mantled pistillate flowers observed here did not. Pseudocarpels of mantled flowers were also devoid of ovary primordia. The stigmatic lobe and the style were heavily vascularised in both normal and mantled samples and showed polyphenol accumulation.

Limited number of male samples were available for analysis. Organogenesis was found to occur at predicted stage 2 in staminate flowers of normal male inflorescences. Normal staminate flowers develop anthers following perianth organs like ASFs. Pollen sacs develop inside the anther lobs producing microsporocytes within. The microsporocytes undergo meiotic division to give rise to pollen tetrad and the haploid male gametophyte, pollen grains. At the same time, tapetum degenerates (Section 7.3.6). Meiotic stages were observed at predicted stage 4. Correctly identifying organogenesis and meiotic stages in staminate flowers are key in the collection of haploid samples for cytogenetic studies and cloning.

Optimal reproductive development is an important yield component in oil palm, like many other crops. Mantled somaclonal variant is associated with aberrant reproductive development and sterility. The improvements made in the tissue-culture process, sequence driven analysis of differential expression of genes and the methylation depended early detection method have been partial victories in eliminating the mantled phenotype. A more methodical study of the basic biology of mantled phenotype as is undertaken here could be a step towards a more comprehensive solution.

CHAPTER 8
FUNCTIONAL QUALITY ASSESSMENT OF NORMAL AND MANTLED OIL PALM POLLEN

8.1. INTRODUCTION

Objectives:

- Establishment of protocols for assessment of the functional quality of oil palm pollen and comparison of the functional quality of pollen samples from normal and mantled sources.

The quantity and quality of pollen grains produced by a flower is an important component of fitness. Many terms such as germinability, viability, vitality, vigour, stainability, and fertility have been used in scientific literature to describe the functional quality of pollen or different aspects of it. 'Pollen viability' signifying the proportion of viable pollen in the pollen load is the most popular attribute among researchers. Lincoln, Boxshall *et al*. (1982) defined pollen viability as "the capacity to live, grow, germinate or develop" (Dafni and Firmage 2000).

Conversely, abortion of pollen grains during pollen formation and development may be termed 'pollen sterility' and the ability of the pollen grains to germinate in a receptive stigma, fertilise ovules and set seed may be called 'fertilization ability'. Furthermore, the capacity of pollen to retain its functionality over an extended period of storage may be termed 'longevity'. Assessment of all these factors gives a much clearer picture of functional quality of pollen (Dafni and Firmage 2000).

8.1.1. Assessment of Pollen Functional Quality

There are different methods for the assessment of the functional quality of pollen grains. Histochemical approaches are commonly adopted for assessing the pollen load, sterility, and viability. These typically utilise the ability of certain chemicals to stain specific constituents of the vegetative cell of the pollen grain (for example: Alexander's test). Conversely some approaches specify activity of enzymes (for example: FCR test). Staining methods are quick and thus most suitable for routine screening of many samples (Chang *et al*., 2014).

Alexander's test (Alexander, 1969) is a widely used, simple and rapid method to assess the functional quality of angiosperm pollen and gymnosperm microgametes. During susceptible stages of pollen development, such as meiosis in pollen mother cells, abortion may occur due to abiotic stresses. Alexander's staining distinguishes the healthy and aborted/immature pollen grains by colour under light microscope.

Fuchsin acid in the staining solution stains acidophilic structures in the cytoplasm, for example: mitochondria and collagen, magenta-red. Orange G also serves as a cytoplasmic stain and improves differentiation. Malachite green is used as the counterstain. It is a weakly basic blue-green dye and binds to the cellulose in the pollen cell wall. As a result, cytoplasm of functional pollen grains, which has fully developed to anthesis, are stained magenta-red. In contrast, the aborted pollen grains, that failed to reach maturity, appear light blue-green as only the walls are stained (Alexander, 1969). Thus according to the categorisation adopted by Dafni and Firmage (2000), this gives a measure of pollen sterility. Despite the popularity and

ease of this method, there are no previous studies quoting its use for oil palm pollen until now.

The fluorochromatic reaction (FCR) test is an easy, quick and accurate method for assessing pollen viability (Li, X., 2011). It can also be used as a vitality indicator for cultured plant cells and some seeds. The method as initially proposed by Heslop-Harrison (1984) tests for esterase activity and membrane integrity. Viable grains when placed into a solution of fluorescein diacetate (FDA) fluoresce under microscopic examination and could be distinguished.

FDA is a non-polar, non-fluorescent fatty acid ester of fluorescein. It freely enters the living cells where it is rapidly hydrolysed by esterase enzymes to liberate fluorescein. Fluorescein is a polar fluorescent molecule. In the presence of an intact plasma membrane, it is retained in the living cells. Fluorescein in healthy cells is thus observable as green fluorescence in blue light under florescent microscope. Tandon (2007) used FCR test to assess and compare pollen viability at storage and after transport and cryopreservation. In this study the applicability of this method is examined for desiccated pollen samples stored at 4°C.

8.1.2. Examination of Germinability and Pollen Tube Growth

Germination tests involve germinating the pollen on the stigmas of emasculated flowers (in vivo) or on artificial media to determine fertilization ability by visual assessment of pollen tube growth. Germination test is routinely employed at research stations to ensure the viability of stored pollen before its use in artificial controlled pollination. However, under

suboptimal conditions viable pollen grains may fail to germinate and fertilize (Chang *et al*., 2014).

Structural and cytochemical aspects of pollen pistil interaction in *Tenera* hybrid was reported in 2001 (Tandon *et al*., 2001). Chemical localisation of stigmatic exudates and microscopy were utilised to study in vivo interactions. Aniline blue fluorescence method was used for *in vitro* examination of pollen germination and pollen tube growth. In the case of successful pollination, pollen grains were found to germinate within 2 hours. Pollen tubes grow down the stigmatic lobe and enter the style via the stigmatic grove (5 hours after pollination) and eventually the locules of the ovary (18 hours after pollination).

Germinability is directly related to ability of the pollen to fertilize, hence it is an important aspect to study and compare under different treatments (For example increases in temperature, water stress, and different methods of pollen handling, storage, and disinfection). For effective comparisons a standardised germination media and method is required. Tandon (2007) employed a germination medium containing sucrose (2.5%), boric acid (100 ppm) and poly-ethylene glycol (10%, MW 10,000) to evaluate pollen germinability before and after cryopreservation. Here, a simple and rapid method for *in vitro* examination of pollen germinability and pollen tube growth is proposed, for pollen samples stored at 4°C.

8.1.3. Significance of Studying Pollen

Pollen grains are the male reproductive cell of a plant and thus are crucial in the normal reproduction process. Pollen grains are easily collected and stored for a considerable length of time. Its culture is simple and rapid, which makes it an ideal system for fundamental and applied research

(Shivanna and Rangaswamy 2012). Exciting upcoming areas of pollen studies include its role in germplasm preservation via cryopreservation and creation of genetic variability via mutation. Standardised methods for pollen assessment will be crucial for these.

Most of our understanding of anther and pollen development is from a limited number of species such as *Arabodopsis*. Techniques including live-cell imaging, using green fluorescent protein reporter genes to monitor expression patterns of multiple genes and proteins involved in anther development, has been achieved in *Arabidopsis*. But extrapolating these results to oil palm is difficult, even with the available technology (Wilson *et al*., 2009; Furness and Rudall, 2001).

Oil palm pollen has played an important role in settling disputes of the crop's origin and spread, via archaeological records. Further, the insect pollinators of oil palm have been widely studied in almost all oil palm growing tracts since successful pollination is an important limiting factor in fruit production in oil palm (Barfod *et al*., 2011; Meléndez and Ponce, 2016; Li *et al*., 2019). But it is important to note that low pollination rates leading to poor fruit set could be caused by either reduced pollination by pollinators or low pollen quantity or both (Tandon *et al*, 2001). It is interesting and significant here that to date, there were very few studies regarding the functional quality of oil palm pollen and none at all about pollen from mantled sources until now.

8.2. MATERIALS & METHODS

8.2.1. Pollen Samples

Dura and *Tenera* pollen samples were obtained from Oil Palm Research Institute- Council for Scientific and Industrial Research (OPRI-CSIR), Ghana and Oil Palm Breeding Research Station, Advanced Agriecological Research Sdn. Bhd. (AAR), Malaysia, respectively (Table 8.1). Optimisation of pollen functional quality tests were achieved using samples 1 to 8 (Table 8.1).

Table 8.1 List of Pollen Samples. Samples are denoted by the sample ID of different *Dura* progeny for rows 1 to 5 and Clone ID/Palm Number and Phenotype (N- Normal, M- Mantled) of *Tenera* ramets for rows 6 to 11. Oil palm Variety and source of pollen samples are specified. OPRI and AAR stands for Oil Palm Research Institute, and Advanced Agriecological Research Sdn. Bhd respectively.

Sl. No	Sample	Oil Palm Variety	Source
1	1680	*Dura*	OPRI-Ghana
2	899	*Dura*	OPRI-Ghana
3	1750	*Dura*	OPRI-Ghana
4	1968	*Dura*	OPRI-Ghana
5	1960	*Dura*	OPRI-Ghana
6	A202-722/8 N	*Tenera*	AAR-Malaysia
7	A204-932/68 N	*Tenera*	AAR-Malaysia
8	A218-933/46 N	*Tenera*	AAR-Malaysia
9	A202-722/61 M	*Tenera*	AAR-Malaysia
10	A204-932/33 M	*Tenera*	AAR-Malaysia
11	A218-933/34 M	*Tenera*	AAR-Malaysia

Dura pollen samples (Table 8.1 -samples 1 to 5) were used to monitor pollen germinability loss during control of *Fusarium oxysporum f. sp. elaeidis* spores in oil palm pollen via UV light exposure [Related study conducted with Dr Kwasi Adusei-Fosu, results available at Adusei-Fosu (2017)].

Pollen samples from normal and mantled *Tenera* ramets belonging to clones A202, A204 and A218 (Table 8.1 -samples 6 to 11) were used for comparative study of pollen health. The clonal lineage of these clones is summarised in Table 8.2.

Table 8.2 Genetic Background of Pollen Sources. Clone lineage is represented by letter 'A' denoting a primary clone followed by a serial number. Genetic background is shown in terms of the parental lines of the ortet palm. In a cross the left is the maternal parent and right the paternal parent (pollen donor).

Clone lineage	Genetic Background
Lineage A202	*Dumpy Deli x Cameroon*
Lineage A204	*Deli x AVROS*
Lineage A218	*Deli x* unknown

All pollen samples were collected by bagging the male inflorescence prior to anthesis. Collected pollen was desiccated on silica and stored at -4°C for up to 6 months. Pollen samples were frozen at -20°C for long term storage.

8.2.2. Alexander's Test

Pollen health was assessed using Alexander's stain (Alexander, 1969). Pollen samples stored at 4°C were used directly or after 15 minutes incubation at 38ºC. The pollen grains were transferred to a drop of Alexander's staining solution (For recipe see Appendix 5) on a glass slide, using a needle. It was covered with a coverslip and observed under a light microscope (Leica Microsystems, UK).

The number of aborted, degenerating and healthy pollen were recorded based on differential staining. Aborted pollen appeared empty with green walls, degenerating pollen appeared pink and flaccid and normal healthy pollen appeared dark purple with densely stained cytoplasm. The percentages were calculated as follows. Partial/ incomplete pollen grains in the microscopic field were excluded from the counts.

$$\textbf{\textit{Percentage of aborted pollen}} = \frac{\text{Number of Aborted Pollen}}{Total} \text{x}100$$

$$\textbf{\textit{Percentage of degenerating pollen}} = \frac{\text{Number of Degenerating Pollen}}{Total} \text{x}100$$

$$\textbf{\textit{Percentage of healthy pollen}} = \frac{\text{Number of Healthy Pollen}}{Total} \text{x}100$$

Standard deviation was calculated between technical replicates.

$$\textbf{\textit{Standard deviation (σ)}} = \sqrt{\frac{\Sigma(X-\mu)^2}{n}}$$

Where X is the value of observation, μ is mean and n is the number of observations

8.2.3. FCR Test

The fluorochromatic reaction (FCR) test was assessed for its applicability in testing pollen viability of oil palm pollen samples. The FCR test brings to light the esterase activity and the membrane integrity of the sample. Viable pollen grains exhibit fluorochromatic reaction (fluoresce under microscopic examination) when tested.

BK buffer S15 MOPS (pH 7.5) was prepared fresh (for recipe see Appendix 5). To 1 ml of the buffer 1 µl of fluorescein diacetate (FDA) stock solution (2 mg FDA/ml of acetone stored at -20 °C) was added. A drop of the FDA-buffer mixture was taken on a slide cleaned with alcohol. Pollen grains were added to the drop using a needle (Li, X., 2011). The slides were visualised under an optical microscope (Leica DM 5000B, Leica, UK) using a fluorescence filter block for blue excitation (exciting fluorescence at 495 nm). Images were processed using inbuilt image analysis software (Leica, UK).

8.2.4. Pollen Germination Test

Pollen germination was examined at different combinations of sucrose and boric acid concentrations (Table 8.3). Pollen samples were added to 500ul

each of the germination media taken in small culture plates, using a needle. The plates were incubated at 28 degree Celsius for 2 hours. Photographs were taken at 40x magnification under a light microscope.

Table 8.3 Composition of the Different Germination Media. The different germination media used in the trial differed in the concentrations of sucrose and boric acid in their composition as detailed.

Treatment	Concentration of Sucrose (% w/v)	Concentration of Boric Acid (ppm)
A	10	0
B	10	500
C	10	1000
D	5	0
E	5	500
F	5	1000

Percentage of pollen germination was calculated as:

$$\textit{Percentage Germination} = \frac{\text{number of pollen tubes}}{\text{total number of pollen}} \times 100$$

8.2.5. Statistical Analysis

All statistical analyses of data were carried out using Genstat 64-bit Release 18.2 (PC/Windows 8) Copyright 2016, VSN International Ltd. The percentages of aborted, degenerating and healthy pollen were analysed by χ^2 test for goodness of fit.

8.3. RESULTS AND DISCUSSION

8.3.1. Functional Quality Determination of Oil Palm Pollen

Alexander's test for Pollen sterility was found to be a simple and effective method for the assessment of oil palm pollen functional quality. Viable pollen grains-stained magenta-red and aborted pollen grains stained blue green. Furthermore, degenerating pollen grains could also be distinguished as they appeared pink and flaccid (Figure 8.1). Incubating the pollen at 38ºC for 15 minutes was found to hasten the absorption of stain by pollen grains (Figure 8.1 B, D), for quicker observations. However, if this staining method is used alongside germination or *in vitro* growth assays, the higher temperature incubation may be avoided since it may reduce viability.

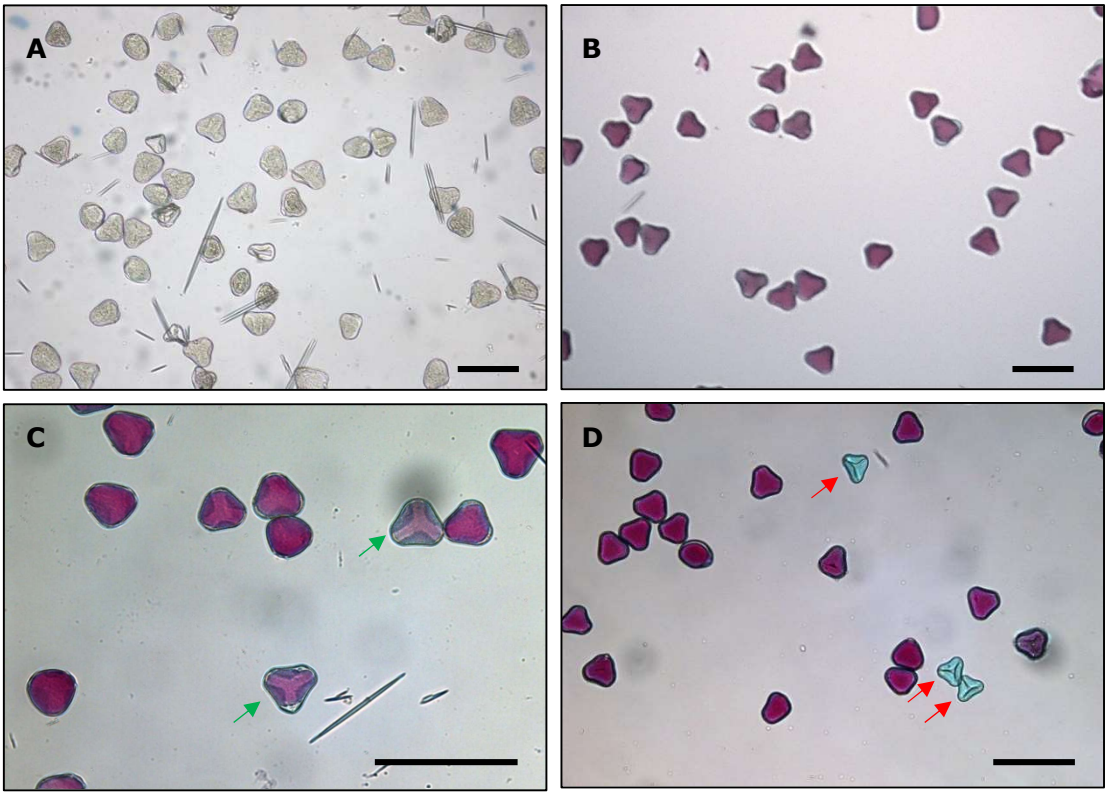

Figure 8.1 Alexander's Test for the Assessment of Oil Palm Pollen Health. (A) shows unstained oil palm pollen grains. (B) is oil palm pollen stained with Alexander's stain observed after 45 minutes at room temperature. (C) and (D) show oil palm pollen stained with Alexander stain observed after 15 minutes of incubation at 38ºC. Incubation accelerated the staining of pollen grains. Healthy pollen was stained magenta red and appear plump. Green arrows indicate degenerating pollen grains which appear flaccid with cell membrane pulling away from the cell walls and have lower intensity of staining. Red arrows indicate aborted pollen grains, which appear empty as only the cell walls are stained (blue-green). The bar represents 100µm.

8.3.2. FCR Test for Pollen Viability

Applicability of FCR test was tested for oil palm pollen. The viable pollen grains showed fluorescence (FCR+) (Figure 8.2) under blue excitation (wavelength = 495 nm). The number of viable pollen observed was considerably less compared to Alexander test. This is in line with previous studies where Alexander staining has been shown to overestimate the pollen functional quality (Frescura *et al*., 2012). The reason for this may be that mature pollen at varying degrees of degradation could be stained the same as healthy grains in Alexander test. However due to time constrains and non-availability of pollen samples this could not be further explored in the present study.

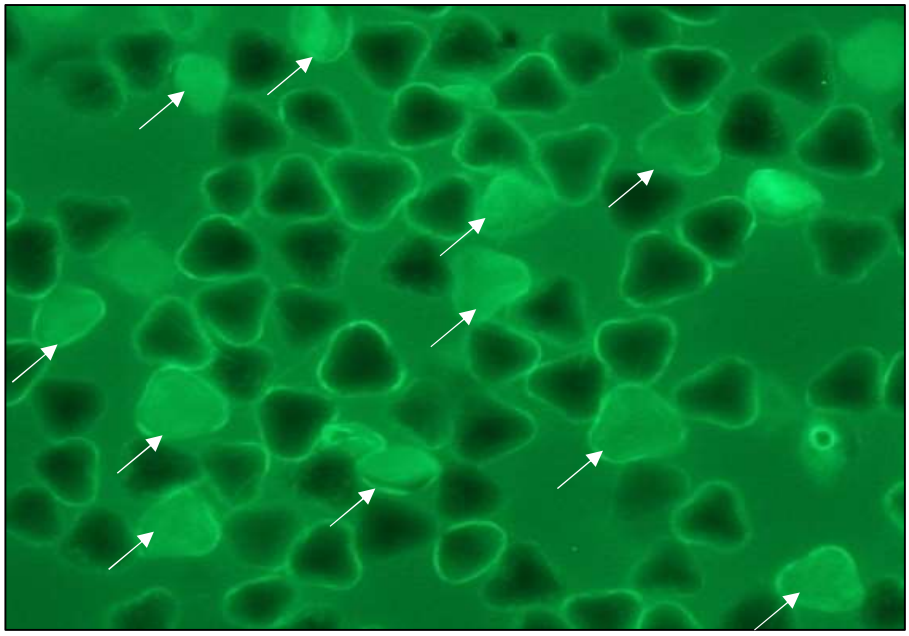

Figure 8.2 Oil Palm Pollen Stained with FDA. Pollen grains stained with FDA were viewed under a florescent microscope with blue excitation. Viable pollen grains indicated by white arrows showed green fluorescence (FCR+) due to the formation of Fluorescein. Fluorescein is formed by the hydrolysis of FDA, which is catalysed by esterase enzymes found in healthy cells.

8.3.3. Pollen Germination Tests

Oil palm pollen germination was examined at different combinations of sucrose and boric acid concentrations (Table 8.3). Treatment E, five percent sucrose solution with 500 ppm boric acid showed maximum germination (Table 8.4 Figure 8.3). Based on this result standardised germination tests (using media containing 5 % sucrose solution with 500 ppm boric acid) for *Dura* pollen samples was proposed, the temperature and treatment time were also controlled in addition to media concentration.

Table 8.4 Effect of Germination Media on Pollen Germination. The trial was conducted using pollen samples from a *Dura* palm. The different germination media differed in their composition: Treatments A, B and C contained 10% (w/v) sucrose and 0, 500 and 100 ppm of Boric acid respectively. Treatments D, E and contained 5% (w/v) sucrose and 0, 500 and 100 ppm of Boric acid respectively. The composition of germination media had an effect on germination of pollen grains. The percentage of germination was calculated based on microscopic examination of treated pollen. Treatment E containing five percent sucrose solution with 500 ppm boric acid showed maximum germination.

Treatment	Percentage Germination (%)
A	61.82
B	34.78
C	45.31
D	55.71
E	87.10
F	21.28

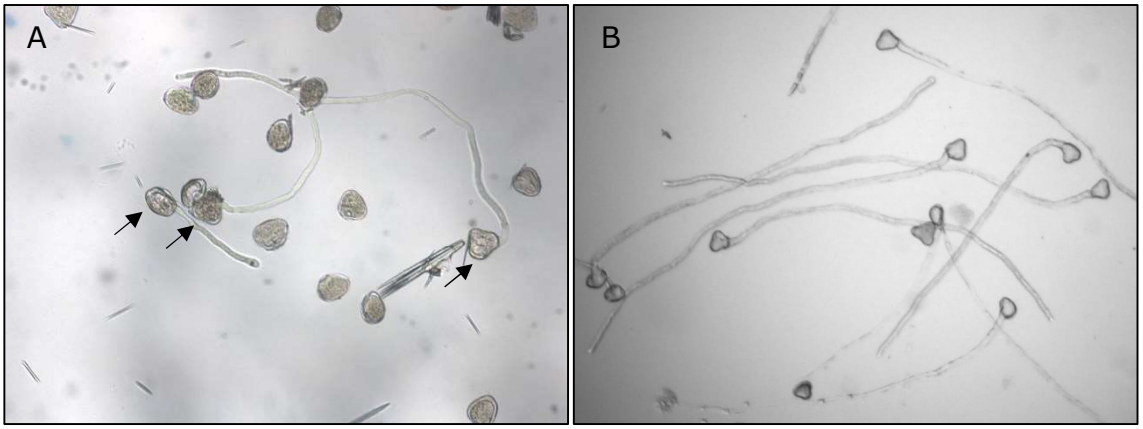

Figure 8.3 Oil Palm Pollen Samples Showing Low (A) and High (B) Germination. Oil palm pollen samples treated with different germination media were incubated at 28^{0}C for 2 hours and observed under a light microscope. Germinating pollen grains (indicated by arrows, in image A) were recognizable by the visible growth of pollen tubes.

The standardised germination test was used to monitor pollen germinability loss during control of *Fusarium oxysporum f. sp. elaeidis* spores in oil palm

pollen via UV light exposure [Related study conducted with Dr Kwasi Adusei-Fosu, results available at Adusei-Fosu (2017)].

Pollen is now used as a germplasm exchange material between countries that grow oil palm. The transmission of *F. oxysporum* via commercial freeze-dried oil palm pollen (Flood *et al*. 1990) is a major concern. Efforts to remove fungal contamination by fungicide treatment of oil palm pollen showed partial success. The potential of UV treatment for eradication *F. oxysporum* from oil palm pollen was considered. High Intensity Pulsed Polychromatic or conventional UV at doses of 60 KJ rendered the fungal spores inactive but this was found to be lethal to the pollen as well [Related study conducted with Dr Kwasi Adusei-Fosu, results available at Adusei-Fosu (2017)].

8.3.4. Pollen Abortion and Degeneration in Mantled Samples

Pollen samples from normal and mantled palms belonging to three different clones were analysed by Alexander's test. In addition to complete abortion of pollen grains, where the pollen grains appear empty, showing only the green counterstain on the pollen walls, varying degrees of degeneration were observed among the samples. The degenerating pollen was distinguished from healthy pollen by the amount of staining and appearance. While healthy pollen grains stained bright purple and appeared turgid, the degenerating pollen grains had milder staining and appeared flaccid (Figure 8.1).

The percentage of aborted, degenerating and healthy pollen was analysed by χ^2 test for goodness of fit. The deviance ratio and χ^2 value showed a

significant difference between normal and mantled samples for all three parameters (Table 8.5).

Two out of the three clones showed higher abortion in the mantled samples (Table 8.5 A, Figure 8.4 A). Clone 932 with the highest rate of abortion among the clones, showed an inverse association, with a slightly lower percentage of aborted pollen in the mantled sample. In this case, the mantled palm had 22.19% pollen abortion in contrast to 23.81% in the sample from the normal palm, with a standard error of 0.19. Nevertheless, it may also be noted that the samples from clone 932 had a standard deviation of 15.05 in the case of mantled and 8.39 in the case of normal whereas the other two clones had standard deviations less than 2.5, among the technical replicates. So, the variation may be at least partially due to human error in sample preparation.

Table 8.5 Accumulated Analysis of Deviance of Percentage of Aborted(A), Degenerating (B) and Healthy pollen (C). Goodness of fit analysis was conducted between mantled and normal pollen samples from 3 different oil palm clones. Within a clone, the phenotype had a highly significant effect on each parameter of pollen health as indicated by the Chi2 probability <.001.

A. Percentage of Aborted Pollen

Source of Variation	Degrees of Freedom	Deviance	Mean Deviance	Deviance Ratio	Chi^2 Probability
Clone*Phenotype	5	784.40	156.88	156.88	<.001
Residual	55	221.67	4.03		
Total	60	1006.07	16.77		

B. Percentage of Degenerating Pollen

Source of Variation	Degrees of Freedom	Deviance	Mean Deviance	Deviance Ratio	Chi^2 Probability
Clone*Phenotype	5	231.52	46.30	46.30	<.001
Residual	55	196.61	3.58		
Total	60	428.13	7.14		

C. Percentage of Healthy Pollen

Source of Variation	Degrees of Freedom	Deviance	Mean Deviance	Deviance Ratio	Chi^2 Probability
Clone*Phenotype	5	717.93	143.59	143.59	<.001
Residual	55	320.77	5.83		
Total	60	1038.69	17.31		

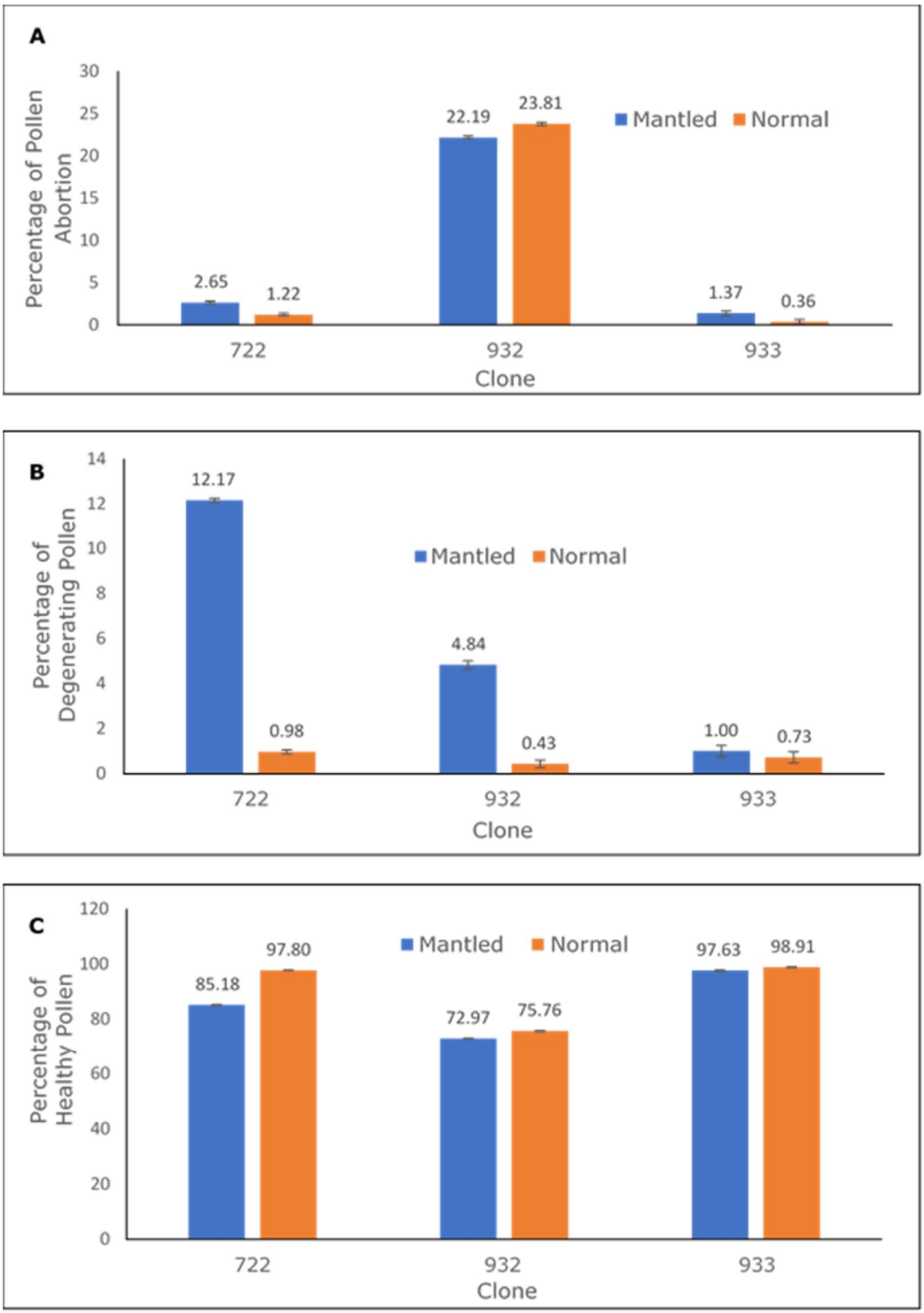

Figure 8.4 Percentage of Aborted(A), Degenerating (B) and Healthy Pollen (C) in Mantled and Normal Pollen Samples. Data is shown for 3 different oil palm clones. In comparison with their normal counterparts, mantled samples from clones 722 and 933 scored poorly in all three pollen health parameters, they had higher percentages of pollen abortion and of pollen degeneration and lower percentage of healthy pollen. Mantled palms of clone 932 had a higher percentage of pollen degeneration and lower percentage of healthy pollen but a slightly lower pollen abortion compared to the normal sample form the same clone. Error bars show standard error.

In the case of the amount of degenerating pollen grains found in the samples, all the three clones showed significantly higher degeneration in their mantled samples. The differences between the percentage of degenerating pollen were 11.19±0.09%, 4.49±0.17% and 0.27±0.25% between the mantled and normal samples from the clones 722, 932 and 933 respectively (Figure 8.4 B). The differences within a clone had a χ^2 probability <0.001, indicating a highly significant deviance ratio (Table 8.5 B).

Percentage of healthy pollen was significantly higher in the case of normal samples in all the clones (χ^2 probability <0.001). It was highest in the case of clone 933, 98.91% in normal and 97.63% in mantled with standard error 0.19. Clone 722 showed the highest disparity between the mantled and normal pollen samples. The amount of healthy pollen in the mantled sample of clone 722 was 12.63% lower than its normal counterpart (Table 8.5 C, Figure 8.4 C).

The data suggests a possible difference in pollen health between samples from normal and mantled palms, which may be indicative of genetic differences that may be associated with pollen developmental regulation. pollen viability in somaclonal variants have been previously reported in *Torenia fournieri* Lind. (Sun *et al*., 2013). Similarly, Kuznetsova *et al*. (2005) detected completely sterile pollen in pea plants (*Pisum sativum* L) regenerated from long-term callus cultures. However, without a larger sample set with biological replicates, concrete conclusions cannot be drawn. Nevertheless, histochemical approaches, such as Alexander staining can be used for the purpose of assessment of pollen health and abortion.

8.4. SUMMARY

Natural hybridisation is oil palm is associated with shortcomings like short lifespan of pollen, loss of pollen quantity, poor synchronisation between pollen shed and stigma receptivity and above all, the production of hybrids with doubtful yields. Consequently, controlled pollination is necessary to produce improved oil palm seedlings and breeders need a quality pollen stock for successful hybridisation.

In vitro germination method is adapted for routine screening of oil palm pollen samples for breeding programmes. However, the accuracy and reproducibility of these methods are dependent heavily on the optimisation of the germination medium and temperature. Alternatively, histochemical staining methods could also be used for pollen quality assurance alone or in combination with germination tests. Here, two histochemical staining methods, Alexander staining for pollen sterility and FCR test for pollen viability, and germination conditions were optimised for oil palm pollen.

Despite the significance of mantled abnormality, pollen samples from mantled palms have not been studied previously. Here, samples from normal and mantled palms were analysed, and pollen health in the same was characterised. A significant difference in pollen abortion, degeneration and health were observed (χ^2 probability <0.001). The Effect of mantled phenotype on pollen health warrants further exploration.

CHAPTER 9
INVESTIGATING THE EFFECT OF MANTLED ON SEX DETERMINATION IN OIL PALM

9.1. INTRODUCTION

Objectives:

- Comparison of the number of male inflorescences born by normal and mantled clones to examine the possible effect of mantled on sex determination.

In oil palm, the same palm tree yields both male and female inflorescences in alternating cycles. The mechanism behind sex determination in oil palm is unclear; nevertheless, the effects of multiple environmental factors have been documented (Adam *et al*., 2011). The sex ratio of a palm, that is the proportion of female inflorescences among the total inflorescences produced by the palm, is an important yield attribute. Years of targeted selection/breeding have reduced the length of the male phase in oil palms to increase the production of female flowers per palm and in turn, more economic oil palm bunches per area. However, adverse environmental conditions have been found to trigger the male phase in palms (Rival, 2017; Woittiez *et al*., 2017).

There is no evidence for a sex chromosome or sex-related loci in oil palm. Differential expression of multiple genes, each regulating specific processes are likely to contribute to the sex-specific features of an inflorescence (Adam *et al*., 2011). Accordingly, sex specific expressions of several genes have been explored (Ho *et al*., 2016, Ong *et al*., 2020).

In the initial investigations of this study Mantled palms were observed to enter the male phase less often than their normal counterparts belonging

to the same clone. This was a previously unreported trait, whereas environmental regulation of sex determination in oil palm has been well documented (Adam *et al*., 2011). It was observed that in younger mantled palms (five years of age) in the screening nursery that were under environmental stress due to close planting did not enter the male phase as in the case of normal palms in the same plot. They were either producing fertile fruit bunches with reduced supernumerary carpels or stopped bearing inflorescence altogether (field observations). It has been suspected that environmental cues can also affect the expression of mantled phenotype, like others under epigenetic control (Thiebaut *et al*., 2019). From the organ-specific RNA sequencing completed by Ooi *et al*. (2019), it is now known that several genes involved in stress response and redox regulation, for example, heat shock protein (HSP) genes, STI-like, BAG molecular chaperons, and ascorbate oxidase are down-regulated in mantled floral organs. The suppression of these genes may also have a part to play in sex determination, which is sensitive to environmental stresses.

Census data (Appendix 7) suggested that severely mantled palms seldom bore male inflorescences (F14 and beyond). It was thus hypothesised that mantled phenotype had a secondary effect on the sex ratio of palms. This hypothesis was tested using field sampling data. The numbers of male and hermaphrodite inflorescences borne by mantled palms and normal palms belonging to the same clone, growing under the same environmental conditions were compared. The field sampling data (Appendix 6) was further characterised with respect to the leaf stages at which mantled male inflorescences were present. Data suggests a possible influence of mantled on sex determination and points to sex specific expression of the phenotype, both of which have not been reported before.

9.2. MATERIALS & METHODS

9.2.1. Data Collection

Census data was collected prior to sampling of the palms, the leaf stage and the sex of the inflorescence were recorded for each palm (Appendix 7). Analysis was done using field sampling data (as described in Chapter 5, section 5.2.3) collected from genotyped and phenotyped palms selected for inflorescence sampling (Appendix 6).

9.2.2. Data Analysis

Comparisons were conducted between normal and mantled palms of the same clone that were sampled together. The Percentage of hermaphrodite and male inflorescences sampled was calculated as follows:

***Percentage of Hermaphrodite and Male Inflorescence* (%HM)** $= \frac{\mathrm{nH} + \mathrm{nM}}{N}$ x100

Where n_H and n_M are the number of hermaphrodite inflorescences and the number of male inflorescences in the sampling range respectively and N is the total number of inflorescences sampled.

The means were calculated for each clone. Mean values were supplemented with standard deviation where applicable, as follows:

***Standard Deviation (σ)* =** $\sqrt{\frac{\Sigma(X-\mu)^2}{n}}$

Where X is the value of observation, μ is mean and n is the number of observations.

Goodness of fit analysis was carried out using Genstat 64-bit Release 18.2 (PC/Windows 8) Copyright 2016, VSN International Ltd.

9.3. RESULTS AND DISCUSSION

9.3.1. Effect of Mantled on Sex Determination

While searching for "Mantled male" in the field, it was noted that mantled palms entered the male phase less frequently than normal palms. This hypothesis was analysed using field data from 4 clones (Table 9.1, 9.2 Figure 9.1), two mature clones namely A229 and R291 and two young clones namely A366 and A478. The percentage of hermaphrodite and male inflorescences sampled from normal and mantled palms of the same clone at the same time were analysed. Sex ratio (number of bunches per harvest cycle) was not used as a parameter as data collected related to developing inflorescences and not bunches. Further, it was not possible to estimate if all the female inflorescences would have reached maturity. Being a floral abnormality that results in sterility, early abortion of inflorescences in mantled palms also could not be discounted.

Mantled palms of clones A229 and A366 did not bear male flowers at all in the sampling range. Samples from one mature mantled palm belonging to R291 included one hermaphrodite and one male inflorescence while the other two mantled palms of the same clone only had female inflorescences. In R291, the male and hermaphrodite inflorescences in the mantled palm were at the end of the non-destructive sampling range. Whether they would have reached maturity or would have been aborted in the course of development is unclear. The normal palms belonging to the same clones all had male inflorescences in the sampling range.

Table 9.1 Number of Female, Hermaphrodite and Male Inflorescences Sampled. Data is presented for palms belonging to mature and young clones, in terms of their sampling groups, where normal and mantled palms under the same environmental conditions were sampled together. Phenotype is based on visual scoring of bunches. The sex of inflorescence at each leaf stage is indicated by the letters F, M and H for Female, Male and Hermaphrodite. X indicates a missing data point. The female phase is highlighted in green and the male phase in yellow. Percentages of Hermaphrodite and Male inflorescences (%HM) indicates a lower incidence of male phase in mantled palms.

Sampling Group	Clone	Palm	Phenotype	Sex of Inflorescence at Each Leaf Stage																					%HM
				F0	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20	F21	
Mature Palms																									
1	A229	71	Normal									F	F	M	M	F	M	F	F	F	F	F	F			25.00
		69	100% Mantled									F	F	F	F	F	F	F	F	F	F	F				0
2	R291	15	Normal								F	F	F	F	F	F	F	M	M	M	F	F				25.00
		19	100% Mantled								F	F	F	F	F	F	F	F	F	F	F	F				0
3		16	Normal										M	M	F	F	F	F								28.57
		23	100% Mantled										F	F	F	F	F	F								0
4		18	Normal								M	F	F	F	F	F	F	F	F	X	M	M				27.27
		17	100% Mantled								F	H	M	F	F	F	F	F	F	F	F	F				16.67
Young palms																									
5	A366	7553	Normal			F	F	M	F	M	X	F	F	F	F	F	F	F	F	F	F	M				18.75
		7551	53% Mantled		F	F	F	F	F	F	F	F	F	F	F	F	F	F	F							0
6		7536	Normal				F	F	F	F	F	F	F	F	F	F	F	F	F	F	F					0
		7554	55% Mantled	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F							0
		7558	44% Mantled	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F						0
7	A478	7838	Normal				F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F			0
		7842	96% Mantled				F	F	F	F	F	F	F	F	F	F	F	H	M	M	F	F	F	F	F	15.79
8		7841	Normal				F	F	F	F	F	F	F	F	H	M	M	M	M	F	F	M	M	M		40
		7839	67% Mantled					M	M	F	F	F	F	F	F	F	F	F	F	F	M	H	M			31.25

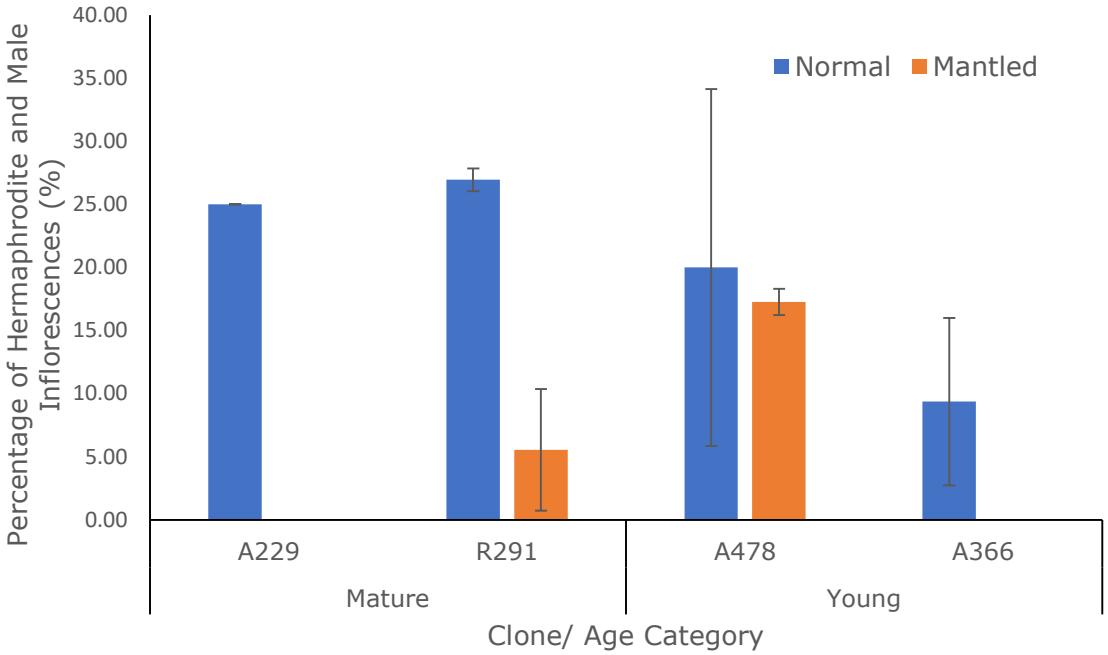

Figure 9.1 Average Percentage of Hermaphrodite and Male Inflorescences (%HM). The proportion of hermaphrodite and male inflorescences obtained in the field sampling of normal and mantled palms belonging to mature and young clones is depicted as percentage values (%HM). The data shown are the average values for all the palms sampled within a clone. Error bars show standard deviation indicative of the variation between palms belonging to the same clone. In all clones, both mature and young, the mantled palms had a lower %HM compared to the normal palms of the same clone. Therefore, data suggests mantled palms enter the male phase less frequently than their normal counterparts.

Clone A478 showed an unusual pattern. The two mantled palms belonging to A478 had the greatest number of male inflorescence samples. Further, one of the normal palms of A478 did not have any male or hermaphrodite inflorescences during sampling. That is the palm did not enter the male phase at all for over 12-18 months, which is uncommon.

In the case of young palms, the sampling range considered extended till F0 as sex was identifiable as early as F0 (Table 9.1). However, young palms have a higher probability of reversion owing to their age and lower severity of mantled phenotype; hence the availability of the male phase may also be an indication of reversion to a normal phenotype.

Table 9.2 Goodness-of-fit Analysis of %HM. Data from normal and mantled palms belonging to the same clone were analysed with regard to the proportion of hermaphrodite and male inflorescences produced (%HM) within the sampling ranges. Within a clone, the phenotype (mantled/normal)of the palms was found to have a significant effect on the percentage of hermaphrodite and male samples, as indicated by the highly significant Chi^2 probability <0.001.

Source of Deviance	Degrees of Freedom	Deviance	Mean Deviance	Deviance Ratio	Chi^2 Probability
Clone*Phenotype	7	222.15	31.74	31.74	<.001
Residual	9	104.89	11.65		
Total	16	327.05	20.44		

Goodness-of-fit analysis of collected samples showed that the phenotype of the palm (normal/mantled), as well as the clonal origin, has a significant effect on the incidence of male inflorescence. Clearly, there are differences between clones and between palms. More significantly, mantled palms enter the male phase less frequently compared to their normal counterpart in this data set (χ^2 probability <0.001) (Table 9.2).

However, the wider applicability of results needs to be validated. Seventeen palms from 4 clones, sampled and studied here are too few in terms of genetic structure. In a perennial tree crop like oil palm, achieving a significant sample size can be challenging. But due to the highly heterogeneous nature of oil palm validation of data in bigger sample sets is necessary to avoid erroneous conclusions owing to individual variations.

If valid, the significant effect of mantled phenotype on sex determination indicates the early manifestation of mantled. Sex determination in oil palm occurs earlier (before F0) than organogenesis (roughly F7). According to previous reports the abnormal expression of *EgDef* in mantled that results in the characteristic visible phenotype, occurs at organogenesis. Hence the abnormal expression of *EgDef* does not account for the possible effect of mantled phenotype on sex determination.

Our current understanding of the mantled phenotype does not provide a clear picture of the upstream effects of *MANTLED* locus. It is thus unclear whether the genes involved in the origin of phenotype or the ones affected by the phenotype or other genes affected by the altered methylation status of mantled palms also affect sex determination in the abnormal palms.

Breeding for an optimum sex ratio, under environmental stresses, is of paramount importance in the context of global climate change. If sex specific expression of mantled phenotype holds true for larger sample sizes, mantled may serve an important role in unravelling the genetic control underlying sex determination in oil palm. This can provide a new avenue to address abiotic stress tolerance in oil palm.

9.4. SUMMARY

The genetic mechanism of sex determination in oil palm has long evaded the scientific community. The sex ratio of the female to male inflorescences on each palm is important for breeding and commercial production. Goodness of fit analysis confirmed that mantled palms entered the male phase less frequently compared to normal counterparts, in the current sample set. The molecular mechanism behind this effect of mantled phenotype on sex determination is unclear.

This discrepancy in the incidence of the male phase could be due to an unknown upstream effect of the *MANTLED* locus earlier in development, or a different epigenetic/genetic effect associated with the phenotype which is directly or indirectly (through differential response to environmental cues) affecting sex determination. Results suggest the need for further research.

Mantled is a unique homeotic mutant and if its effect on sex determination holds true for larger sample groups, it may play a key role in unravelling the mechanism of sex determination in oil palm. This could be of theoretical and practical significance for breeding as well as evolutionary studies.

CHAPTER 10
CONCLUSION

The present study explores the reproductive development of a crucial crop for the future, the African oil palm - *Elaeis guineensis*. The contribution of this PhD research is primarily a detailed understanding of the spatio-temporal sequence of events in inflorescence development in normal and mantled palms and novel methods for the assessment of mantled bunches and oil palm pollen.

Oil palm has a major global impact - economically, socially and environmentally. Economically, oil palm offers high oil yields and extensive uses unmatched by any other oil crop (Koh and Wilcove, 2007, Fitzherbert *et al*., 2008). Socially, it is the main driver of socio-economic change in South-East Asian countries such as Malaysia. Environmentally, the unsustainable expansion of oil palm plantations has caused negative impacts on biodiversity and sustainability (Khatun *et al*., 2017; Rival, 2017; 2018).

A sustainable increase in palm oil production is possible through scientific intervention to improve productivity and policy intervention to improve sustainability (Mayes *et al*., 2008, Woittiez *et al*., 2017). Micro-propagation via tissue culture helps the production of uniform superior planting material that offers a definite yield advantage over hybrid seeds and creation of novel genetic variants by modern genetic techniques such as genetic transformation and genome editing via CRISPR/Cas 9 system (Kushairi *et al*., 2010; Bortesi and Fischer, 2015; Woittiez *et al*., 2017; Weckx *et al*., 2019; Yarra *et al*., 2019). However, limitations related to current tissue culture practices, including the high incidence of deleterious somaclonal

variations (primarily the floral abnormality named "mantled") hinder effective deployment of clonal progeny (Kushairi *et al*., 2010; Weckx *et al*., 2019).

Mantled abnormal phenotype manifests as the homeotic transformation of floral whorls, parthenocarpy and loss of oil yield (Jaligot *et al*., 2011; Ong-Abdullah *et al*., 2015). Global hypomethylation, single-sequence methylation polymorphisms and expression polymorphism were detected in association with Mantled. However, these were not useful for the early detection of mantled palms, because of the very high individual and genotype-dependent variations in expression levels and methylation (Jaligot *et al*., 2011). The *KARMA* test based on the hypomethylated genomic region associated with the mantled phenotype has been a promising solution (Ong-Abdullah *et al*., 2015, 2016, 2018; Ishak *et al*., 2020; Sarpan *et al*., 2020). However, the precise origin and genetic and epigenetic mechanisms involved in the formation of mantled phenotype are still unknown.

In this thesis, it was hypothesised that a systematic morphological and histological analysis of oil palm reproductive development in normal and mantled ramets may reveal novel effects of the mantled phenotype. Accordingly, eight research objectives were set which were investigated in chapters 3 to 9.

Firstly, methods for the selection of ramets for comparative studies of normal and mantled phenotypes were presented. Chapter 3 explored the various considerations in the selection of palms to ensure the stability of the phenotypes under investigation and availability of a good range of developmental stages. Further, difficulties encountered in the field and

measures to minimise the environmental effects of data are discussed. The plant material thus selected consisted of 52 clonal palms from major breeding populations. The palms selected belonged to five clones from two age categories - mature (ten years in the field) and young (three years in the field).

To ensure within and between clone comparisons, the genotypic and phenotypic identities were confirmed using SSR fingerprinting and morphological and molecular phenotyping respectively. SSR fingerprinting utilised 20 SSRs, with good discriminatory power between clones, to develop unique fingerprints. The genotyping method was found an efficient and effective way of culling out the off-types and supports the findings of Singh *et al*., (2007) and Chee *et al*., (2015) regarding the applicability of SSRs for fingerprinting oil palm clones.

Phenotypic identity (normal or mantled) of the ramets were determined by morphological characterisation via phenotyping of unripe fruit bunches and molecular characterisation via *KARMA* assay. As no standardised method for quantifying mantled severity was available a new phenotyping protocol was designed. The protocol employs the scoring of individual fruits of the entire bunch in the case of young palms or 20 selected spikelets in the case of mature palms. Scoring data was used to calculate the mantled percentage (%M) which proved useful for comparisons.

Methylation detection on the *Karma* transposon at the *EgDEF1* locus using *Rsa*I (*KARMA* assay; Ong-Abdullah *et al*., 2015, 2016, 2018) was conducted in technical triplicates. The *KARMA* assay revealed three outliers - two false positives (mantled palms showing a high percentage of CHG methylation) and one false negative (normal palms showing low methylation

percentage). It is possible that the false-negative may be due to reversion. Such errors in the *KARMA* test were pointed out by Weckx *et al*. (2019) as well. This warrants further investigation. The reason for the false positives was unclear but upholds the necessity for morphological characterisation. But it may be noted that in the present study the analysis was limited to only the *Rsa*I restriction site, and it was not possible to analyse the outliers by bisulphite sequencing (Ong-Abdullah *et al*., 2015). From the results obtained here, it is proposed that multiple detection techniques (morphological and/or molecular) may be combined in future investigations to reduce errors in the determination of mantled phenotype.

Further characterisation of mantled phenotype is described in chapter 4, wherein the heterogeneity or variability of phenotype in homeotic transformation (number of pseudocarpels per fruit) and fertility (development of kernel) was examined. This was achieved through visual scoring and calculation of specific parameters that reflect on the severity and variability of the abnormality. Mantled percentage denoting the severity of the phenotype was analysed alongside the weighted mean of the number of pseudocarpels (PC $Mean_{wt}$) representing the average degree of homeotic transformation and the fertility in mantled fruits (%FM). The extent of homeotic transformation was found to be positively correlated to the severity of abnormality (correlation coefficient 0.92). As expected, fertility in fruits decreased with the severity of mantled (correlation coefficient of -0.77), but fertility was found to be highly variable within bunches and also between bunches of the same palm.

The variability of homeotic transformation was captured by calculating the percentage distribution of fruits with different numbers of pseudocarpels

and fertility across these categories. Previous publications had employed a subjective categorisation of mantled phenotype (Jaligot *et al*., 2000; Shearman *et al*., 2013; Ong-Abdullah *et al*., 2015). Even when the categories accounted for the severity of the phenotype the heterogeneity was not accounted for. Evidence suggests different variations of mantled phenotype may have different expression profiles (Yaacob *et al*. 2013). The phenotyping regime and mathematical parameters presented here could quantify the heterogeneity and phenotypic complexity of mantled phenotype in addition to the severity (%M). The results confirmed the assumption that mantled phenotype is less stable among young ramets.

An interesting aspect to look at in future studies will be the effect of the phenotype on the inflorescence architecture and by consequence yield parameters. During phenotyping in some of the clones, more fruits per spikelet were found in unripe mantled bunches. MADS-box genes have been found to influence inflorescence architecture and patterning (Schilling *et al*., 2018). Plus, the use of epigenetic variations and homeotic mutations for their altered inflorescence structure for agronomic improvements and economic profits are not unheard of (Vaitkūnienė *et al*., 2019, Latutrie *et al*., 2019). Phyllotaxis of male and female spikelets on oil palm inflorescence was reported in 1970 (Thomas *et al*., 1970) but this has not yet been investigated in the light of this epigenetic homeotic mutant. However, investigation of this was beyond the scope of this project. However, the spikelet sampling and scoring regime proposed in chapters 3 and 4 will be suitable for such analyses.

Chapter 5-7 focuses on methods of extraction and examination of oil palm inflorescence samples. Preliminary observations were all in line with the

previous report by Adam *et al*., (2005). Oil palm inflorescences develop deep within the crown of the palm at the base of developing leaves over a period of 2 to 3 years. Male and female flowers are produced in separate inflorescences. Occasionally at the transition of female and male phases, hermaphrodite inflorescences are produced. The inflorescence sampling protocols for the non-destructive sampling in mature palms and destructive sampling in young palms were optimised through trial. Inflorescence sampling in oil palm for research purposes is time-consuming and labour intensive. Optimised protocols of chapter 5 ensured effective and efficient extraction of samples from palms. These inflorescence sampling techniques proposed may also be adopted for molecular studies.

Frond number (also referred to as leaf stage) was considered as the primary field reference in previous studies (Adam *et al*., 2005; Ong-Abdullah *et al*., 2015). However, the developmental stage at a certain frond number depends on the frequency of frond emergence, and so a certain frond number does not always indicate a specific developmental stage. Inflorescence length was evaluated across the sampling range for its effectiveness as a field reference for the inflorescence developmental stage. Length of inflorescence was a more accurate indicator of developmental progression among samples of the same palm, compared to the frond number, and was useful in checking the numbering of fronds. However, it was affected by the age of the palms, sex of the inflorescences and to a certain degree the mantled phenotype. The young palms produced smaller inflorescences, but within the age category, there was only a negligible difference between clones. Hermaphrodite and male inflorescences were longer (not considering the length of peduncle) compared to female inflorescences at the same leaf stage, an observation not reported

previously. The difference with respect to phenotype (mantled/normal) was limited to certain leaf stages and showed high variability across sample groups. Hence the length of inflorescence was found to be an unsuitable reference of developmental stage in this study. Accordingly, a more detailed characterisation of inflorescence samples for developmental classification was adopted as described in chapter 6.

The histology protocol was adapted from previous literature (Adam *et al*., 2005, Sarpan *et al*., 2015) and was optimised through trials (Chapter 5). The protocol was examined in its entirety for the different types of samples used. Fine sectioning of samples to a size below 2.5cm and collection of samples in fixative in the field itself reduced the amount of fixative required, improved the effectiveness of fixation, and reduced discolouration in samples. Two different fixatives, PF and GPC, were compared. The difference in fixative type did not cause a significant difference in the quality of slides produced, hence the simpler (a smaller number of chemical components in the recipe and easier preparation) of the two was selected for histology protocol. Older flower samples (closer to anthesis) were too hard even after extended butanol treatment and sometimes broke off from the resin during sectioning. It is proposed that Paraplast X-tra resin which reports better resin penetration (de Farias *et al*., 2018) may be investigated as an alternative for these stages. For the purposes of this study, the histology protocols presented here were found to be adequate.

To ensure reasonable comparisons the samples of equivalent stages of development had to be identified in normal and mantled palms. Chapter 6 examines the reproductive developmental process through the developmental stages obtained. The previous model of developmental stage

prediction (Sarpan *et al*., 2015) was fine-tuned with supplementary visual staging to account for key stages of development. The samples collected from selected palms were classified into developmental categories using the previously published predictive algorithm, visual characterisation and histological study. The reproductive developmental process observable through destructive and non-destructive sampling was classified into eight developmental categories based on the predictive algorithm and field observations. Early inflorescence development in young inflorescences was further classified into four using histology.

The different developmental categories were validated using ANOVA (F probability<0.001) and Fisher's protected least significant difference test. Fisher's LSD test shows mean separation between mature and young palms and not phenotype. The effect of mantled phenotype on length of inflorescence was further analysed within developmental categories and was found to be not significant. Hence the inconsistent differences observed between normal-mantled sampling groups at a few leaf stages in certain clones was deemed coincidental.

The developmental classification was characterised to allow easy prediction of developmental stages. The initial categorisation into eight developmental categories is possible in the field itself to identify comparable samples, as it relies on the length of inflorescence and macro features observable by the naked eye. An interesting future application of these may be in tissue culture. Oil palm inflorescences are a potential source of meristematic tissue for tissue culture. While oil palm only has only one vegetative meristem (SAM) there is an infinite number of generative flower meristems in each inflorescence. Reversion of inflorescence tissue for meristem cultures has

been successful in coconut palm (Weckx *et al*., 2019). Similar efforts are underway in oil palm, and if achieved could lead to the development of a novel propagation technique in oil palm (Zulkarnain *et al*., 2019; Panggabean *et al*., 2021). The sampling techniques proposed in chapter 5 and the developmental classification described in chapter 6 can help determine the appropriate level of maturity of inflorescences and their extraction to be used as explant.

A detailed histological analysis of oil palm inflorescence samples was conducted through high-resolution microscopy to pinpoint morphological and physiological changes during inflorescence development at the cellular level. A comparative reproductive developmental series from early inflorescence development to floral maturity thus developed is presented in Chapter 7. The series enables comparisons between age categories (young vs mature), sexes as well as phenotype (normal vs mantled) across the developmental categories. As previously reported in oil palm (Adam *et al*., 2005) and related palm species *Elaeis oleifera* (de Farias *et al*., 2018) and *Cocos nucifera* (Perera *et al*., 2010) the majority of the developmental period was the slow expansion of the inflorescence structure and the final 1/3rd of the developmental period was the rapid development of floral organs of individual flowers (organogenesis). Sex of inflorescence was identifiable in developmental category 1D (development of floret primordia), where the difference in the morphology of male and female spikelets in the inflorescences was evident.

Comparative analyses were conducted between normal and mantled samples as well as mature and young clones within the framework of the newly defined developmental categories. Microanatomy of young

developing female inflorescence revealed a near-identical developmental process in all samples. No differences were observed in the reproductive development in mantled samples until organogenesis. In the developmental category Floral Triad 3 mantled phenotype was identifiable by micro-morphology as pseudocarpels (also referred to as supernumerary carpels) were distinguishable. One of the key observations made was regarding the Abortive Staminate Flowers (ASFs) of floral triads in female inflorescences. Contrary to previously published data (Adam *et al*., 2005), in the present set of samples, the ASFs of mantled floral triads showed normal development. They developed anthers with pollen sacs containing microsporocytes just like their normal counterparts. This is an indication of female specific expression of mantled phenotype in this sample set.

Another possible avenue for future research is on pollen-pistil interaction in normal and mantled flowers. In addition to the developmental characterisation in chapters 6 and 7, the histology and histochemical methods prescribed in chapters 5 and 8 may be useful in this assessment. Previously Tandon *et al*., (2001) had reported the structural and cytochemical aspects of pollen pistil interaction in *Tenera* hybrids, but this has not been explored in relation to mantled abnormality. Whereas similar somaclonal abnormality in date palm *Barhee* cv. has been linked to malformation of stigma and faulty fertilization leading to parthenocarpy (Abd-Elhaleem *et al*., 2020).

In the present study, no mantled male samples could be analysed but normal development of male inflorescences was characterised using the available samples. Classification of stages in male inflorescence development was done using the predictive algorithm alone. Organogenesis

(stage 2) and meiotic stages (stage 4) in staminate flowers were correctly identified (Chapter 5).

The emergence of male inflorescences has been previously associated with the stress response of oil palm (Adam *et al*., 2011; Jaligot, 2018). Hence this opens up a new line of enquiry into the relationship between mantled abnormality and stress response. Previous research shows the downregulation of several genes involved in stress response and redox regulation in mantled floral organs (Ooi *et al*., 2019). Hence, further investigation of 'mantled male' was conducted in chapter 8, where pollen samples from normal and mantled samples were analysed and chapter 9 where the incidence of male inflorescences in normal and mantled palms was compared.

Despite its relevance in breeding and germplasm conservation studies of oil palm pollen are limited (Tandon *et al*., 2007; Myint *et al*., 2012). Histochemical staining methods for assessment of pollen functional quality offer higher accuracy and reproducibility compared to *in vitro* germination methods that are currently adopted for routine screening of oil palm pollen. However, no standard protocols were available for oil palm pollen. So, two histochemical staining methods, Alexander staining for pollen sterility and FCR test for pollen viability, and germination conditions were optimised.

Pollen samples from mantled palms have not been studied previously. In fact, previous reports had suggested that mantled male inflorescence are completely sterile due to homeotic transformation of stamens to carpel-like structures and produced no pollen. This was not the case in our sample set. Male inflorescences of mantled palms had normal morphology. Samples from normal and mantled palms were analysed in terms of pollen functional

quality. A significant increase in pollen abortion and degeneration was observed in mantled samples (χ^2 probability <0.001). The proportion of healthy pollen was significantly less in mantled (χ^2 probability <0.001). In this particular study, the small sample size limited the generalisability of the results. However, male sterility and loss of pollen viability have been reported in association with somaclonal variations in other plants (Kuznetsova *et al*., 2005; Sun *et al*., 2013). So, the effect of mantled phenotype on pollen health warrants further exploration.

The mechanisms behind sex determination and genetic and environmental effects on sex ratio (number of female flowers borne by a palm) have been investigated by many as it is an important yield parameter. The effect of mantled on sex determination is an area not investigated previously. Field observations suggested a lower incidence of male phase in mantled ramets, this was examined using field sampling data. Goodness of fit analysis confirmed that mantled palms entered the male phase less frequently compared to normal counterparts, in the current sample set. That is our results suggest that mantled phenotype may influence sex determination in oil palm. This could be due to an unknown upstream effect of the MANTLED locus earlier in development, or a different epigenetic/genetic effect associated with the phenotype which is directly or indirectly (through differential response to environmental cues) affecting sex determination. This will be an interesting aspect for future studies.

It is expected that future breeding and research in oil palm will rely on not only the fully sequenced genome and genomic tools, but also the available mutants like mantled (Rival *et al*. 2009; Thiebaut *et al*., 2019; Low *et al*.,

2020). Mantled offers a valuable model for studying the homeotic/MADS-box genes, epigenetic machinery and floral development in oil palm.

A large amount of research has already taken place on the mantled abnormality in the past three decades. It is evident that the mantled abnormality has an altered expression of genes involved in floral development including B-class MADS-box gene, *DEFICIENS (DEF)* which is responsible for the characteristic aberrant phenotype. However, our data suggest the abnormality may have far-reaching effects beyond what we understand now, and that these may be occurring earlier than organogenesis. Correspondingly altered expression of *LEAFY (LFY)* an upstream regulator of reproductive development in plants has been associated with mantled (Vetaryan *et al*., 2018). In addition, genes involved in primary hormone responses, DNA replication and repair, chromatin remodelling, and RNA mediated DNA methylation and many others have been found differentially expressed in the mantled mutant (Beulé *et al*., 2008; Rival *et al*., 2009).

As hypothesised the systematic morphological and histological analysis of oil palm reproductive development in normal and mantled ramets revealed two novel effects of the mantled phenotype namely female specific homeotic transformation in floral triads and lower incidence of male phase. Considering the many difficulties involved in the study of perennial palm species like oil palm, and of the highly heterogeneous nature of mantled, standardised, and systematic approaches as proposed in this thesis, will serve useful in future investigations.

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APPENDIX 1

Results of Primer Screening

Primer IDs are as per MPOB and Billotte *et al*. (2005) for primers 1 to 4 and 5 to 24 respectively (Sequence information available in table 3.4). Tm=52°C. Bands are specified by the approximate size of the amplicon and letters to indicate their number and position. For example, 217ab indicates two bands of size 217 were present and 217b indicates only the second band of size 217 was present. All SSRs showed high levels of polymorphism among the 5 control genotypes (not included in the scoring) except for primer pair mEgCIR03376, which showed no amplification (highlighted in grey). Primer pairs sEg00035 and mEgCIR02600 were monomorphic for all three clones (highlighted in red) and were rejected. Twelve primer pairs showed polymorphism between two clones (highlighted in yellow) and 9 were polymorphic between all three clones (highlighted in green). Thus, the first 20 of these 21 polymorphic markers that is, primers 2-12, 14, 16-23 were used for genotyping.

	Primer ID	Size	Clone/Palm					
			R291/ 23	R291/ 16	A299/ 69	A299/ 68	R295/ 27	R295/ 24
1	sEg00035	180	180	180	180	180	180	180
2	sMg00025	217	217ab	217ab	217ab	217ab	217b	217b
3	sMg00042	266	266ab	266ab	266bc	266bc	266bc	266bc
4	sMg00108	205	205ab	205ab	205b	205b	205a	205a
5	mEgCIR00369	225	225a	225a	225bc	225bc	225ac	225ac
6	mEgCIR03428	194	194ab	194ab	194cd	194cd	194bd	194bd
7	mEgCIR03649	303	303bc	303bc	303ab	303ab	303bc	303bc
8	mEgCIR03544	207	207a	207a	207ab	207ab	207ac	207ac
9	mEgCIR02595	203	203b	203b	203ab	203ab	203b	203b
10	mEgCIR03358	227	227abc	227abc	227a	227a	227abc	227abc
11	mEgCIR00783	315	315a	315a	315b	315b	315a	315a
12	mEgCIR03389	112	112ac	112ac	112ab	112ab	112ac	112ac
13	mEgCIR02600	296	296ab	296ab	296ab	296ab	296ab	296ab
14	mEgCIR03808	209	209bc	209bc	209ab	209ab	209b	209b
15	mEgCIR03376	232	X	X	X	X	X	X
16	mEgCIR02332	223	223bc	223bc	223a	223a	223b	223b
17	mEgCIR02492	267	267abc	267abc	267bd	267bd	267cd	267cd
18	mEgCIR03311	195	195a	195a	195ab	195ab	195a	195a
19	mEgCIR02427	135	135ab	135ab	135a	135a	135ab	135ab
20	mEgCIR03546	305	305ab	305ab	305b	305b	305ab	305ab
21	mEgCIR00521	156	156cd	156cd	156ad	156ad	156bc	156bc
22	mEgCIR00177	133	133ab	133ab	133bc	133bc	133ab	133ab
23	mEgCIR03298	156	156c	156c	156a	156a	156ab	156ab
24	mEgCIR0257	305	305b	305b	305ac	305ac	305b	305b

APPENDIX 2

Results of Genotyping

Table 1. Genotyping results for palms belonging to clones A229, R291, R295, A366 and A478, based on primer sets 1 to 10 (following table 3.4). Primer IDs are as per MPOB and Billotte *et al*. (2005) for primers 1 to 3 and 4 to 10 respectively. Sample names include clone/palm and phenotype (N- Normal and M- Mantled). Results show bands observed in terms of their size expressed as the number of base pairs (bp). The unique fingerprint of the clone is stated on top in terms of the size of bands expected (bp). Off types, with different fingerprints to the rest of the clone, are highlighted in yellow.

Primer ID / Sample name	sMg00025	sMg00042	sMg00108	mEgCIR00369	mEgCIR03428	mEgCIR03649	mEgCIR03544	mEgCIR02595	mEgCIR03358	mEgCIR00783
Clone A229										
Size of Bands expected (bp)	**211/218**	**256/260**	**200**	**204/225**	**183/186**	**306/315**	**204/210**	**188/192**	**210**	**322/330**
A229/64 M	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/67 M	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/69 M	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/76 M	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/77 M	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/68 N	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/70 N	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/71 N	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/72 N	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330
A229/73 N	211/218	256/260	200	204/225	183/186	306/315	204/210	188/192	210	322/330

Primer ID / Sample name	sMg00025	sMg00042	sMg00108	mEgCIR00369	mEgCIR03428	mEgCIR03649	mEgCIR03544	mEgCIR02595	mEgCIR03358	mEgCIR00783
Clone R291										
Size of Bands expected (bp)	**211/218**	**252/256**	**190/200**	**200**	**173/180**	**296/306**	**204**	**192**	**210/215/230**	**322**
R291/17 M	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/19 M	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/22 M	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/23 M	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/24 M	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/15 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/16 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/18 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/20 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R291/21 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R291/1 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/2 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/3 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R291/4 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R291/5 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322

Primer ID / Sample name	sMg00025	sMg00042	sMg00108	mEgCIR00369	mEgCIR03428	mEgCIR03649	mEgCIR03544	mEgCIR02595	mEgCIR03358	mEgCIR00783
Clone R295										
Size of Bands expected (bp)	**218**	**256/260**	**194/196**	**200/225**	**180/186**	**296/306**	**204/211**	**192**	**210/215/230**	**322**
R295/20 M	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/21 M	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/26 M	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/27 M	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/28 M	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/22 N	211/218	256/260	174/200	200/235	180/186	296/304	204	192	210	322/323
R295/23 N	211/218	256/260	174/200	200/235	180/186	296/304	204	192	210	322/323
R295/24 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/25 N	211/218	256/260	174/200	200/235	180/186	296/304	204	192	210	322/323
R295/29 N	211/218	256/260	174/200	200/235	180/186	296/304	204	192	210	322/323
R295/1 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/2 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/3 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322
R295/4 N	211/218	252/256	190/200	200	173/180	296/306	204	192	210/215/230	322
R295/5 N	218	256/260	194/196	200/225	180/186	296/306	204/211	192	210/215/230	322

Primer ID / Sample name	sMg00025	sMg00042	sMg00108	mEgCIR00369	mEgCIR03428	mEgCIR03649	mEgCIR03544	mEgCIR02595	mEgCIR03358	mEgCIR00783
Clone A366										
Size of Bands expected (bp)	**211/216**	**252/256**	**190/200**	**200/235**	**173/184**	**294/306**	**211**	**188/192**	**215/230**	**322/330**
A366/7551 M	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7554 M	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7558 M	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7536 N	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7550 N	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330
A366/7552 N	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7553 N	211/216	252/256	190/200	200/235	173/184	294/306	211	188/192	215/230	322/330
A366/7556 N	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330

Primer ID / Sample name	sMg00025	sMg00042	sMg00108	mEgCIR00369	mEgCIR03428	mEgCIR03649	mEgCIR03544	mEgCIR02595	mEgCIR03358	mEgCIR00783
Clone A478										
Size of Bands expected (bp)	**211/218**	**259/260**	**190/198**	**200/204**	**184/186**	**304/308**	**211**	**198/208**	**210/212**	**322/330**
A478/7839 M	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330
A478/7842 M	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330
A478/7838 N	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330
A478/7841 N	211/218	259/260	190/198	200/204	184/186	304/308	211	198/208	210/212	322/330

Table 2. Genotyping results for palms belonging to clones A229, R291, R295, A366 and A478, based on primer sets 11 to 20 (following table 3.4). Primer IDs are as per Billotte *et al*. (2005). Sample names include clone/palm and phenotype (N- Normal and M- Mantled). Results show bands observed in terms of their size expressed as the number of base pairs (bp). The unique fingerprint of the clone is stated on top in terms of the size of bands expected (bp). Off types, with different fingerprints to the rest of the clone, are highlighted in yellow.

Primer ID / Sample name	**mEgCIR 03389**	**mEgCIR 03808**	**mEgCIR 03376**	**mEgCIR 02332**	**mEgCIR 02492**	**mEgCIR 03311**	**mEgCIR 02427**	**mEgCIR 03546**	**mEgCIR 00521**	**mEgCIR 03298**
A229										
Size of Bands expected (bp)	**40/50**	**188/192**	**221/223**	**180/216**	**265/272**	**192/195**	**108**	**278**	**138/161**	**132**
A229/64 M	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/67 M	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/69 M	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/76 M	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/77 M	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/68 N	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/70 N	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/71 N	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/72 N	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132
A229/73 N	40/50	188/192	221/223	180/216	265/272	192/195	108	278	138/161	132

Primer ID / Sample name	mEgCIR 03389	mEgCIR 03808	mEgCIR 03376	mEgCIR 02332	mEgCIR 02492	mEgCIR 03311	mEgCIR 02427	mEgCIR 03546	mEgCIR 00521	mEgCIR 03298
R291										
Size of Bands expected (bp)	**40/55**	**192/206**	**215**	**220/230**	**260/265 /272**	**195**	**110**	**270/278**	**156/161**	**155**
R291/17 M	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/19 M	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/22 M	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/23 M	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/24 M	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/15 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/16 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/18 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/20 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R291/21 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R291/1 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/2 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/3 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155
R291/4 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R291/5 N	40/55	192/206	215	220/230	260/265/ 272	195	110	270/278	156/161	155

Primer ID / Sample name	mEgCIR 03389	mEgCIR 03808	mEgCIR 03376	mEgCIR 02332	mEgCIR 02492	mEgCIR 03311	mEgCIR 02427	mEgCIR 03546	mEgCIR 00521	mEgCIR 03298
R295										
Size of Bands expected (bp)	**40/55**	**190**	**215**	**180/220**	**278**	**195**	**110**	**270/278**	**148/156**	**132/146**
R295/20 M	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/21 M	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/26 M	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/27 M	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/28 M	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/22 N	40/55	192/206	215	180/230	278	195	110	274/278	138/148	132/146
R295/23 N	40/55	192/206	215	180/230	278	195	110	274/278	138/148	132/146
R295/24 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/25 N	40/55	192/206	215	180/230	278	195	110	274/278	138/148	132/146
R295/29 N	40/55	192/206	215	180/230	278	195	110	274/278	138/148	132/146
R295/1 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/2 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/3 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146
R295/4 N	40/55	192/206	215	220/230	260/265/272	195	110	270/278	156/161	155
R295/5 N	40/55	190	215	180/220	278	195	110	270/278	148/156	132/146

Primer ID / Sample name	mEgCIR 03389	mEgCIR 03808	mEgCIR 03376	mEgCIR 02332	mEgCIR 02492	mEgCIR 03311	mEgCIR 02427	mEgCIR 03546	mEgCIR 00521	mEgCIR 03298
A366										
Size of Bands expected (bp)	**55/62**	**192/206**	**215/223**	**180**	**275/278**	**194/195**	**95/110**	**270/278**	**138/161**	**155**
A366/7551 M	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7554 M	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7558 M	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7536 N	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7550 N	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155
A366/7552 N	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7553 N	55/62	192/206	215/223	180	275/278	194/195	95/110	270/278	138/161	155
A366/7556 N	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155

Primer ID / Sample name	mEgCIR 03389	mEgCIR 03808	mEgCIR 03376	mEgCIR 02332	mEgCIR 02492	mEgCIR 03311	mEgCIR 02427	mEgCIR 03546	mEgCIR 00521	mEgCIR 03298
A478										
Size of Bands expected (bp)	**40/55**	**190**	**221/223**	**180/230**	**272/278**	**197/205**	**110**	**274/278**	**138/161**	**155**
A478/7839 M	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155
A478/7842 M	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155
A478/7838 N	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155
A478/7841 N	40/55	190	221/223	180/230	272/278	197/205	110	274/278	138/161	155

APPENDIX 3

Results of Phenotyping via Visual Scoring

Table 1. Phenotyping results of unripe bunches from palms belonging to clones A229, R291, R295, A366 and A478, based on visual scoring (as described in section 4.2.1). Palm ID is in the format Clone/Palm Phenotype (N= Normal and M= Mantled). Frond number indicated the sequential order of the frond from which the unripe bunch was extracted (numbering as described in section 3.2.2). The age category of the palms and the scoring method used are specified on the top. The total number of fruits and spikelets per bunch, the total number of fruits scored (T), the total number of normal fruits (N), the total number of mantled fruits (M) and the number of unscorable fruits (U) are shown as counts. Percentage abnormality (%M), fertility in mantled (%FM) and sterility in normal (%SN) were calculated for each bunch scored and are expressed in percentages. PC Mode is the most frequently occurring number of pseudocarpels (in the ranges specified) among the fruits of the bunches scored. PC $Mean_{wt}$ is the weighted mean of the number of pseudocarpels in the bunches.

	Palm ID	**Frond No**	**Total fruits**	**Total spikelets**	**T**	**N**	**M**	**U**	**%M**	**%FM**	**%SN**	**PC $Mode_{0-8}$**	**PC $Mode_{1-8}$**	**PC $Mean_{wt}$**
Mature Palms (Scoring of Selected Spikelet)														
1	A229/71 N	NA	NA	NA	100	100	0	0	0.00	0.00	0.00	0	0	0.00
2	R291/15 N	23	NA	NA	258	258	0	0	0.00	0.00	0.00	0	0	0.00
3	R291/18 N	23	NA	NA	288	288	0	0	0.00	0.00	0.00	0	0	0.00
4	R295/24 N	24	NA	NA	393	393	0	0	0.00	0.00	0.00	0	0	0.00
5	A229/69 M	19	NA	NA	350	0	332	18	100.00	0.00	0.00	5	5	5.18

	Palm ID	Frond No	Total fruits	Total spikelets	T	N	M	U	%M	%FM	%SN	PC Mode$_{0-8}$	PC Mode$_{1-8}$	PC Mean$_{wt}$
6	R291/17 M	23	NA	NA	274	0	274	0	100.00	0.00	0.00	6	6	5.59
7	R291/19 M	23	NA	NA	222	0	221	1	100.00	3.62	0.00	6	6	4.97
Young Palms (Scoring of Whole Bunch)														
8	A366/7553 N	23	334	49	334	334	0	0	0.00	0.00	0.00	0	0	0.00
9	A478/7838 N	25	514	68	514	504	0	10	0.00	0.00	0.00	0	0	0.00
10	A366/7558 M	27	110	43	110	87	21	2	19.44	0.00	0.00	0	1	0.26
11	A366/7558 M	27	242	43	242	67	139	36	67.48	63.31	0.00	0	2	1.33
12	A366/7551 M	25	166	32	166	100	53	13	34.64	66.04	0.00	0	1	0.61
13	A366/7551 M	20	242	33	242	120	103	19	46.19	100.00	0.00	0	1	0.76
14	A366/7551 M	19	174	42	174	35	126	13	78.26	100.00	0.00	2	2	1.94
15	A366/7554 M	24	191	43	191	58	100	33	63.29	67.00	22.41	0	1	1.20
16	A366/7554 M	27	215	46	215	112	95	8	45.89	0.00	0.00	0	1	0.71
17	A478/7839 M	29	328	54	328	179	123	26	40.73	17.07	54.75	0	1	0.85
18	A478/7839 M	26	414	53	414	28	349	37	92.57	44.99	39.29	6	6	3.67
19	A478/7842 M	28	372	65	372	6	338	28	98.26	18.64	33.33	6	6	4.55
20	A478/7842 M	25	81	55	81	5	72	4	93.51	1.39	20.00	6	6	4.87

Table 2. Phenotyping results of unripe bunches from palms belonging to clones A229, R291, R295, A366 and A478, based on visual scoring (as described in section 4.2.1). Palm ID is in the format Clone/Palm Phenotype, where N= Normal and M= Mantled. Frond number indicated the sequential order of the frond from which the unripe bunch was extracted (numbering as described in section 3.2.2). The age category of the palms and the scoring method used are specified on the top. The total number of fruits belonging to each category based on the number of pseudocarpels (supernumerary carpels) present and fertility (F=Fertile and NF=Non-fertile) are shown as counts for each bunch scored.

	Palm ID	Frond No	Number of Pseudocarpels																	
			0 (Normal)		1		2		3		4		5		6		7		8	
			F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF
Mature Palms (Scoring of Selected Spikelet)																				
1	A229/71 N	NA	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	R291/15 N	23	258	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	R291/18 N	23	288	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	R295/24 N	24	393	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	A229/69 M	19	0	0	0	3	0	6	0	28	0	54	0	98	0	89	0	50	0	4
6	R291/17 M	23	0	0	0	0	0	1	0	1	0	23	0	65	0	180	0	3	0	1
7	R291/19 M	23	0	0	2	4	0	3	1	16	1	35	2	63	2	90	0	2	0	0
Young Palms (Scoring of Whole Bunch)																				
8	A366/7553 N	23	334	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	A478/7838 N	25	504	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	A366/7558 M	27	87	0	0	15	0	5	0	1	0	0	0	0	0	0	0	0	0	0
11	A366/7558 M	27	67	0	34	11	32	24	22	12	0	4	0	0	0	0	0	0	0	0
12	A366/7551 M	25	100	0	13	13	14	3	8	0	0	1	0	1	0	0	0	0	0	0
13	A366/7551 M	20	120	0	58	0	29	0	11	0	4	0	1	0	0	0	0	0	0	0

	Palm ID	Frond No	Number of Pseudocarpels																	
			0 (Normal)		1		2		3		4		5		6		7		8	
			F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF
14	A366/7551 M	19	35	0	27	0	41	0	36	0	14	0	8	0	0	0	0	0	0	0
15	A366/7554 M	24	45	13	26	14	23	10	17	8	1	1	0	0	0	0	0	0	0	0
16	A366/7554 M	27	112	0	0	57	0	23	0	15	0	0	0	0	0	0	0	0	0	0
17	A478/7839 M	29	81	98	19	37	2	33	0	18	0	3	0	5	0	2	0	3	0	1
18	A478/7839 M	26	17	11	23	17	29	17	13	42	21	40	9	52	62	3	0	20	0	1
19	A478/7842 M	28	4	2	2	3	10	23	9	33	17	42	16	66	8	89	1	19	0	0
20	A478/7842 M	25	4	1	0	2	0	6	0	8	0	11	0	9	0	14	1	11	0	10

APPENDIX 4

Reference Series

A reference series was prepared using samples obtained through non-destructive sampling of one normal *Tenera* ramet from the nursery. Female inflorescence samples were collected from the leaf stages F9 to F18. Spikelets from the top and bottom of the inflorescence were collected from F14 to F18 and the whole inflorescence was taken from F9 to F13. Samples were packed on ice in the field and transported to the laboratory. Measurements were recorded concerning dimensions and weights of florets and individual organs as summarised in table 1. Figure 1 and 2 show the increase in length and weight over the leaf stages respectively. Plates 1 and 2 show photographs of the samples and the extent of separation possible in the field. This data was used as a guideline for estimating the size of containers and the amount of fixative required for sampling for microscopy and planning the field sampling protocol (Chapter 5). The reference series was also used for establishing visual staging categories (Chapter 6).

Table 1: Length and weight measurements of inflorescences and parts, for leaf stages within the non-destructive sampling range. X indicates data not recorded.

Leaf stage	Length (cm)			Weight (g)				
	Inflorescence	Spikelet	Floret	Floret	Bract	Sepals	Petals	Gynoecium
F18	35	x	2.6	1.2	0.15	0.1	0.095	0.81
F17	34	10.85	2.6	0.56	0.09	0.05	0.05	0.37
F16	34	12.1	2.3	0.42	0.11	0.035	0.026	0.165
F15	33	10.3	0.7	0.07	x	x	x	x
F14	33	9	0.7	0.07	x	x	x	x
F13	25	7.5	0.5	0.02	x	x	x	x
F12	11	3	0.2	x	x	x	x	x
F11	8.5	x	x	x	x	x	x	x
F10	2.5	x	x	x	x	x	x	x
F9	2.5	x	x	x	x	x	x	x

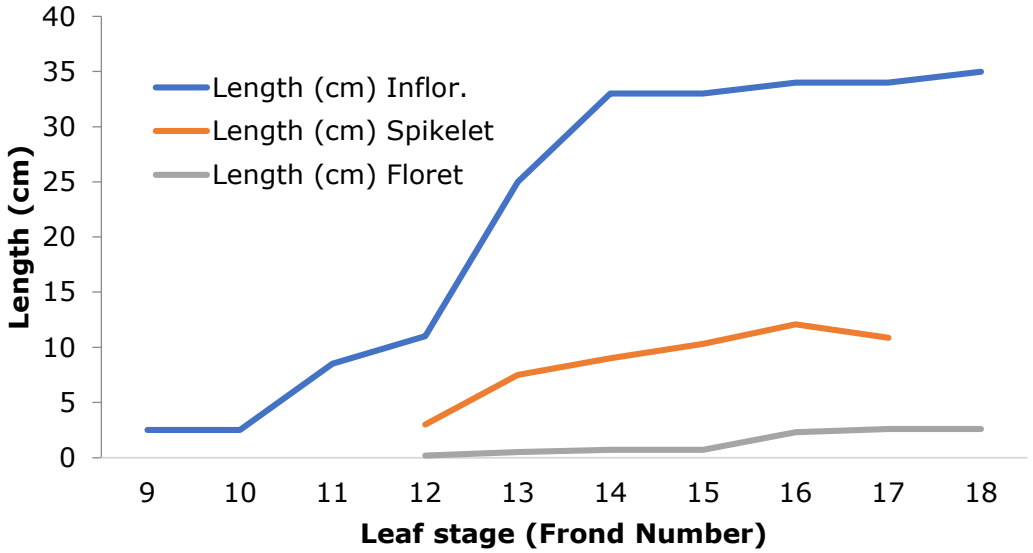

Figure 1 Increase in size through leaf stages. The increase in length of inflorescence, spikelets and individual florets is shown across leaf stages indicated by the number of the subtending leaf.

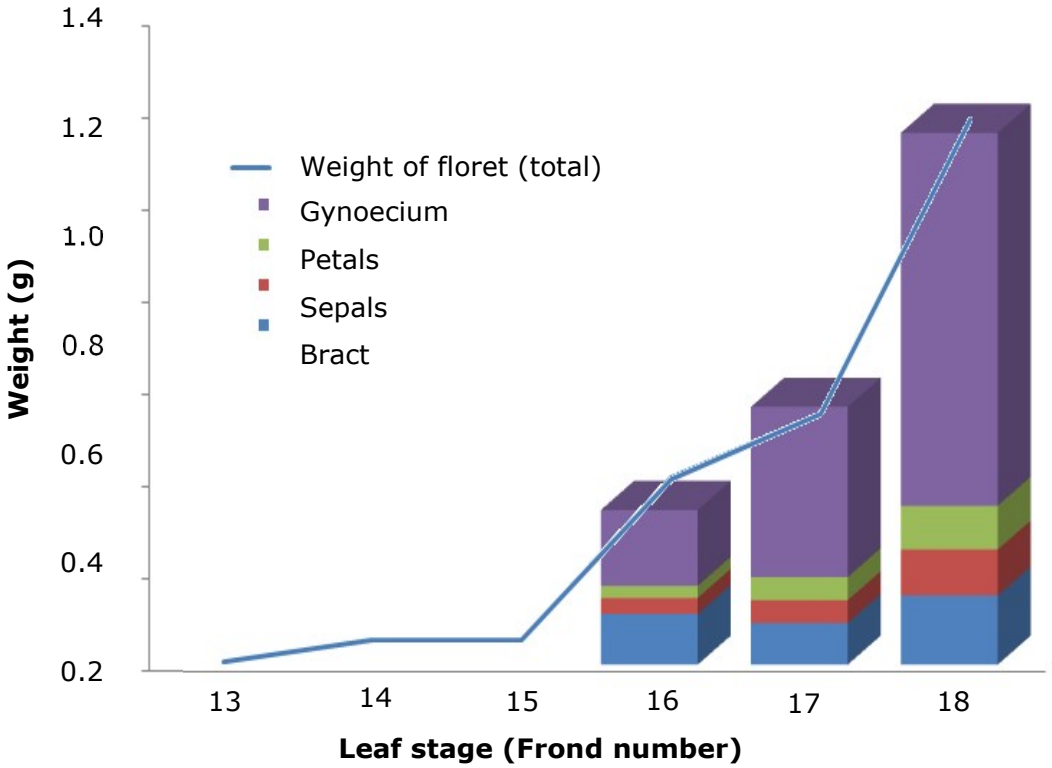

Figure 2 Increase in weight of floret and component organelles through the leaf stages. The increase in weight of individual florets is shown as the line graph. The bar graph indicates the contribution of each floral organelle to the total weight. Data is shown across leaf stages indicated by the number of the subtending leaf.

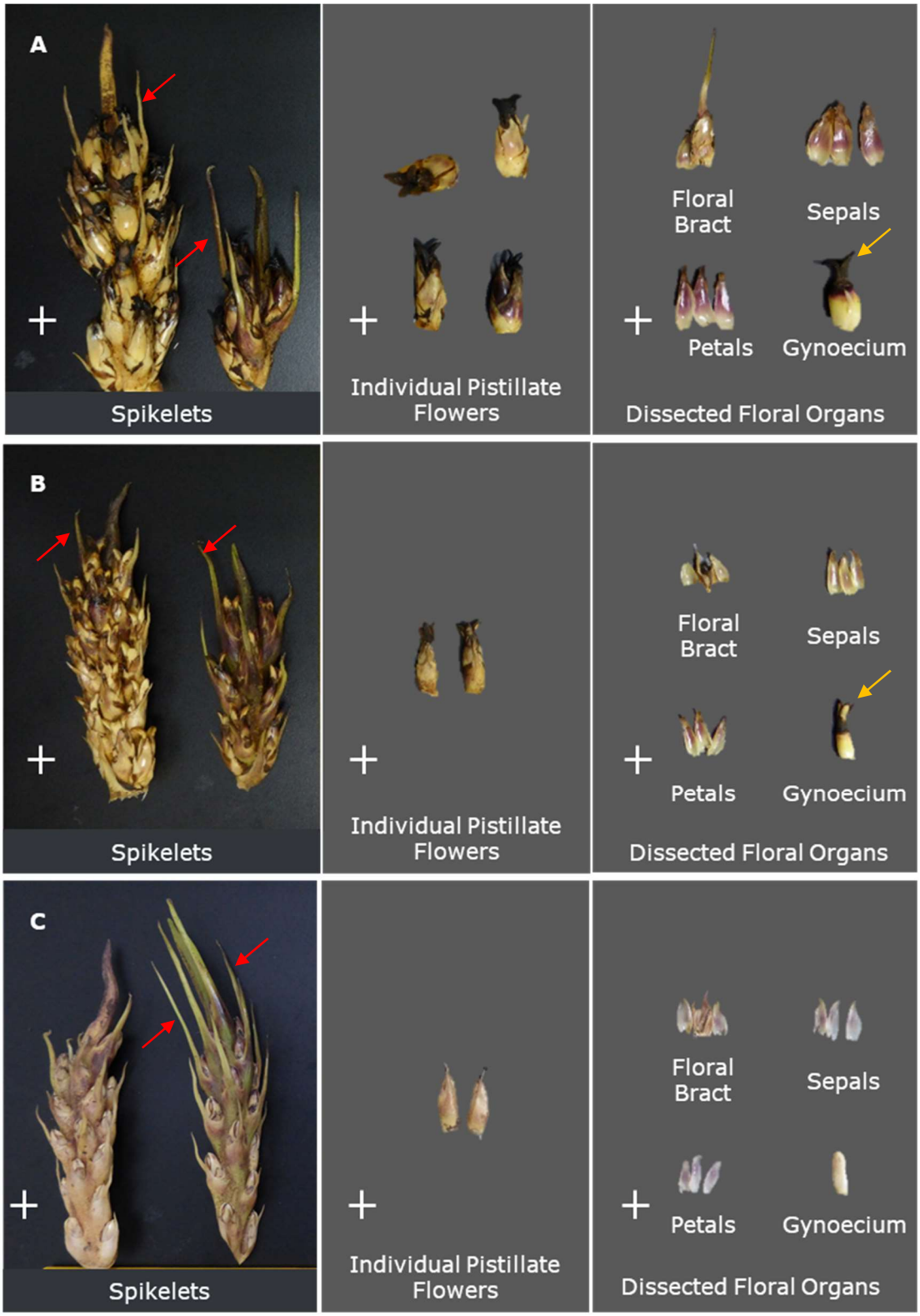

Plate 1: Samples from leaf stages 18 (A), 17 (B) and 16 (C). From the left are spikelets, individual pistillate flowers and dissected floral organs. Scale is indicated by the crossbar, each bar =1cm.
Floral triad bracts (red arrows) are fibrous and spiky. The stigmas are gaping and necrotic in samples from F18 and F17 (yellow arrows) indicating a stage beyond anthesis.

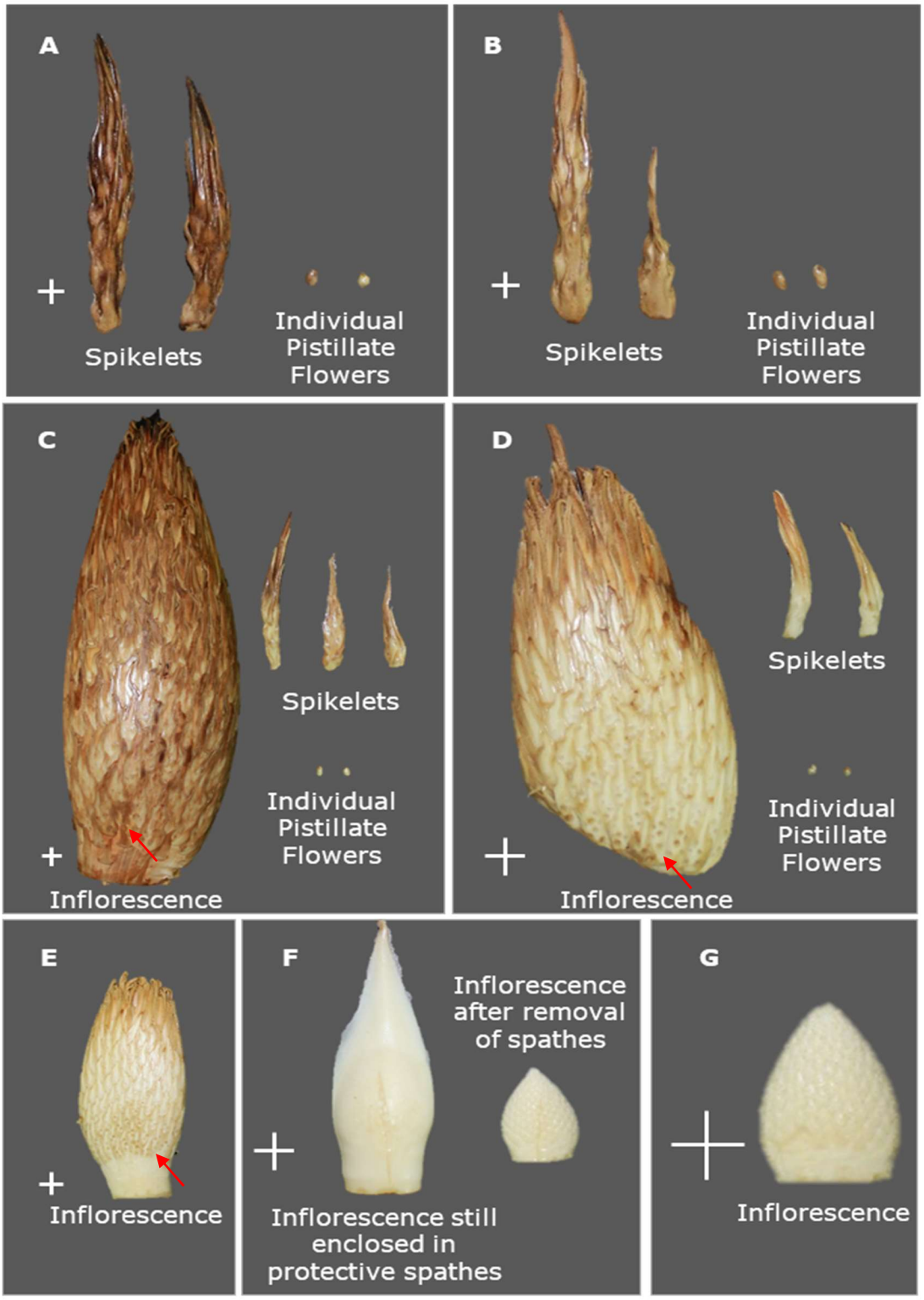

Plate 2: Samples from leaf stages 15 (A), 14 (B), 13 (C), 12 (D), 11 (E), 10 (F) and 9 (G). Spikelets and extracted individual pistillate flowers are shown for samples from F15 and F14. Whole inflorescence, spikelets and extracted individual pistillate flowers are shown for samples from F13 and F12. Whole inflorescences are shown for F11, F10 and F9. Floral triad tissue is visible as spots (red arrows) in F13 (C), F12 (D) and F11 (E). In the case of leaf stage F10 (F) inflorescence before and after the removal of the protective spathes is shown. The scale is indicated by the crossbar, each bar =1cm.

APPENDIX 5

Recipes for Solutions and Stains

A. LI-COR GEL SOLUTIONS

a. Recipe for Gel

Ingredient	Quantity
6% LICOR solution	20ml
TEMED	15µl
10% APS solution	150µl

b. For 600ml of LI-COR gel solution (6%)

Note: To facilitate the dissolving of urea, the solution may be warmed.

Ingredient	Quantity
Urea	252 g
50% long ranger solution (LRGS)	72ml
10X TBE	72ml
distilled-deionized water	

c. For 10ml of 10% APS solution

Note: Store at 4°C.

Ingredient	Quantity
Ammonium persulfate	1g
distilled-deionized water	10ml

B. FIXATIVES

1. Glutaraldehyde-Paraformaldehyde-Caffeine (GPC) Fixative

a. Paraformaldehyde 10%

Weigh 20g of paraformaldehyde (Merk 4005, extra pure). Dissolve in 200ml of distilled water. Stir, with heating at 60-65ºC inside the fume hood to completely dissolve the powder. Add some drops of 1N NaOH to get clear. Cooldown the solution before using. Store at room temperature.

b. Phosphate buffer (pH7.2, 0.2M)

Note: Store in the refrigerator

Solution A	
NaH_2PO_4, anhydrous	2.40g
or NaH_2PO_4*H2O	2.76g
Distilled water	100ml
Solution B	
Na_2HPO_4*12H2O	7.16g
or NaH_2PO_4	2.84g
Distilled water	100ml
Phosphate buffer solution	
Solution A	28ml
Solution B	72ml

c. Caffeine (Sigma C-0750)

Weigh 1g of caffeine inside the hood. Dissolve in 2ml of distilled water with little heating. Dissolve the powder completely.

2. 4% Paraformaldehyde (PF) fixative

a. 10x PBS (Stock)

For 1L 10x PBS, pH 7.2	
Ingredient	**Quantity**
NaCl	74g
Na_2HPO_4	9.94g
NaH_2PO_4	4.14g
Make up the volume with dH_2O	

b. Dissolve 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) by heating in a water bath for 1.5-2 hours. Add 0.1% (v/v) each of Triton X-100 and Tween 20 while still warm.

C. HISTOLOGY STAINS

3. Toluidine blue stain

Note: Stain slides with 0.5% toluidine blue to check tissues.

Na acetate buffer (0.2 M pH 4.6)	
Solution A	
Acetic acid	12 ml
Distilled water	988 ml
Solution B	
Na acetate 3H2O	27.21 g
or anhydrous	16.41 g
Water	950 ml
Mix equal volume of the solutions to make 1L. Adjust pH.	
Dissolve 0.5g of toluidine blue.	

4. Periodic acid (Sigma P7875) 250ml/staining glass

Note: Prepare just before using, DO NOT STORE.

Periodic acid	1g
Distilled water	100ml

5. Schiff's reaction (in the dark)

Dissolve 1g of basic fuchsin in 200ml of boiling distilled water. Mix and let the solution cool down. At 30C, add 2g of disodium metabisulfite per 20ml of 1N HCl. Mix and incubate overnight or one day in a tightly covered flask in the dark. Add 0.5g of neutralized activated carbon. Mix and filter inside the hood. Cover the funnel and the bottle with aluminium foil.

The solution is good as long as it is clear or pale yellow. Keep the solution in the dark in the refrigerator. If water is added to Schiff's reaction and it turns pink, the solution is no longer good.

NOT TO BE RE-USED.

6. **Naphthol blue-black (Sigma N3005)**

7% acetic acid	100ml
(7 ml of glacial acetic acid + 93 ml of H2O)	
naphthol blue-black	1g%

Note: Heat the 7% acetic acid at 60C and add the naphthol blue black while stirring. Could be RE-USED.

D. POLLEN STAINS

1. **Alexander's staining solution**

Ethanol	95%	10 ml
Malachite green	1% in 95% Ethanol	1ml
Distilled water	50 ml	
Glycerol	25 ml	
Phenol	5g	
Chloral hydrate	5 g	
Fuchsin acid	1% w/v solution in water	5 ml
Orange G	1% w/v solution in water	0.5 ml
Glacial acetic acid	depending on thickness of pollen wall	1-4 ml

2. BK buffer S15 MOPS (pH 7.5) for FCR test

Ca $(NO_3)_2.4H_2O$ (MW 236)	30 mg/L (0.127 mM)
$MgSO_4.7H_2O$ (MW 246.5)	20 mg/L (0.081 mM)
KNO_3 (MW 101)	10 mg/L (0.1 mM)
Sucrose	15%
MOPS (MW209)	10 mM (pH 7.5)
Stored at -20 °C	

APPENDIX 6

Field Sampling Data with Event Probabilities and Predicted Stage of Inflorescence Development

The table shows field sampling data which included both details of the palm sampled as well as specifics of the inflorescence samples. Details of the sampled palms including clone, palm number, phenotype (N- Normal, M- Mantled), field planning date, inflorescence sampling date, and calculated age at sampling. Specifics of the inflorescence samples are the number of the frond from which the inflorescence was extracted (frond no), length and sex. The Event probabilities for developmental stages 1 to 4 and the predicted stage of inflorescence development were calculated based on the statistical prediction model proposed by Sarpan *et al*., (2015). Where the number format displays a number in exponential notation, replacing part of the number with E+n, in which E (exponent) multiplies the preceding number by 10 to the nth power.

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
1	A229	71	N	Apr-03	Aug-14	11	19	46	Female	1	1	1	0.999996	>4
2	A229	71	N	Apr-03	Aug-14	11	18	47	Female	1	1	1	0.999998	>4
3	A229	71	N	Apr-03	Aug-14	11	17	47	Female	1	1	1	0.999998	>4
4	A229	71	N	Apr-03	Aug-14	11	16	49	Female	1	1	1	0.999999	>4
5	A229	71	N	Apr-03	Aug-14	11	15	46	Female	1	1	1	0.999996	>4
6	A229	71	N	Apr-03	Aug-14	11	14	34	Female	1	1	1	0.98842	>4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
7	A229	71	N	Apr-03	Aug-14	11	13	17.5	Male	1	0.999996	0.950162	0.001613	3 to 4
8	A229	71	N	Apr-03	Aug-14	11	12	12	Female	1	0.989866	0.04246	4.31E-05	2 to 3
9	A229	71	N	Apr-03	Aug-14	11	11	9	Male	0.999999	0.55692	0.001621	5.96E-06	2 to 3
10	A229	71	N	Apr-03	Aug-14	11	10	5	Male	0.959108	0.003776	1.97E-05	4.27E-07	1 to 2
11	A229	71	N	Apr-03	Aug-14	11	9	4.5	Female	0.853145	0.001832	1.14E-05	3.07E-07	1 to 2
12	A229	71	N	Apr-03	Aug-14	11	8	4	Female	0.58998	0.000887	6.55E-06	2.21E-07	1 to 2
13	A229	69	M	Apr-03	Aug-14	11	18	56	Female	1	1	1	1	>4
14	A229	69	M	Apr-03	Aug-14	11	17	57	Female	1	1	1	1	>4
15	A229	69	M	Apr-03	Aug-14	11	16	38.5	Female	1	1	1	0.999397	>4
16	A229	69	M	Apr-03	Aug-14	11	15	25	Female	1	1	0.999987	0.184686	3 to 4
17	A229	69	M	Apr-03	Aug-14	11	14	15.5	Female	1	0.999936	0.677626	0.000432	3 to 4
18	A229	69	M	Apr-03	Aug-14	11	13	12	Female	1	0.989866	0.04246	4.31E-05	2 to 3
19	A229	69	M	Apr-03	Aug-14	11	12	8	Female	0.99999	0.227529	0.000539	3.08E-06	1 to 2
20	A229	69	M	Apr-03	Aug-14	11	11	6	Female	0.997391	0.015918	5.94E-05	8.25E-07	1 to 2
21	A229	69	M	Apr-03	Aug-14	11	10	5	Female	0.959108	0.003776	1.97E-05	4.27E-07	1 to 2
22	A229	69	M	Apr-03	Aug-14	11	9	4	Female	0.58998	0.000887	6.55E-06	2.21E-07	1 to 2
23	A229	69	M	Apr-03	Aug-14	11	8	3.5	Female	0.262752	0.00043	3.78E-06	1.59E-07	<1
24	A478	7838	N	Jul-11	Oct-14	3	20	20	Female	1	1	0.999999	0.975519	>4
25	A478	7838	N	Jul-11	Oct-14	3	19	18	Female	1	1	0.999994	0.914268	>4
26	A478	7838	N	Jul-11	Oct-14	3	18	18	Female	1	1	0.999994	0.914268	>4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
27	A478	7838	N	Jul-11	Oct-14	3	17	17.5	Female	1	1	0.999989	0.884664	>4
28	A478	7838	N	Jul-11	Oct-14	3	16	14	Female	1	1	0.999477	0.433049	3 to 4
29	A478	7838	N	Jul-11	Oct-14	3	15	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
30	A478	7838	N	Jul-11	Oct-14	3	14	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
31	A478	7838	N	Jul-11	Oct-14	3	13	2.7	Female	0.994517	0.390035	0.007366	0.000445	1 to 2
32	A478	7838	N	Jul-11	Oct-14	3	12	0.9	Female	0.544021	0.044833	0.001019	0.000136	1 to 2
33	A478	7838	N	Jul-11	Oct-14	3	11	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
34	A478	7838	N	Jul-11	Oct-14	3	10	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
35	A478	7838	N	Jul-11	Oct-14	3	9	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
36	A478	7838	N	Jul-11	Oct-14	3	8	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
37	A478	7838	N	Jul-11	Oct-14	3	7	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
38	A478	7838	N	Jul-11	Oct-14	3	6	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
39	A478	7838	N	Jul-11	Oct-14	3	5	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
40	A478	7838	N	Jul-11	Oct-14	3	4	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
41	A478	7838	N	Jul-11	Oct-14	3	3	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
42	A478	7842	M	Jul-11	Oct-14	3	21	20	Female	1	1	0.999999	0.975519	>4
43	A478	7842	M	Jul-11	Oct-14	3	20	20	Female	1	1	0.999999	0.975519	>4
44	A478	7842	M	Jul-11	Oct-14	3	19	20	Female	1	1	0.999999	0.975519	>4
45	A478	7842	M	Jul-11	Oct-14	3	18	18	Female	1	1	0.999994	0.914268	>4
46	A478	7842	M	Jul-11	Oct-14	3	17	16.5	Female	1	1	0.999967	0.798714	>4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
47	A478	7842	M	Jul-11	Oct-14	3	16	21.5	Male	1	1	1	0.990748	>4
48	A478	7842	M	Jul-11	Oct-14	3	15	13	Male	1	0.999999	0.998425	0.283229	3 to 4
49	A478	7842	M	Jul-11	Oct-14	3	14	9	Hermaphrodite	1	0.999832	0.88515	0.027523	3 to 4
50	A478	7842	M	Jul-11	Oct-14	3	13	3.5	Female	0.999409	0.671201	0.01761	0.000754	2 to 3
51	A478	7842	M	Jul-11	Oct-14	3	12	2.3	Female	0.983442	0.263557	0.004752	0.000342	1 to 2
52	A478	7842	M	Jul-11	Oct-14	3	11	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
53	A478	7842	M	Jul-11	Oct-14	3	10	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
54	A478	7842	M	Jul-11	Oct-14	3	9	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
55	A478	7842	M	Jul-11	Oct-14	3	8	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
56	A478	7842	M	Jul-11	Oct-14	3	7	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
57	A478	7842	M	Jul-11	Oct-14	3	6	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
58	A478	7842	M	Jul-11	Oct-14	3	5	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
59	A478	7842	M	Jul-11	Oct-14	3	4	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
60	A478	7842	M	Jul-11	Oct-14	3	3	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
61	R291	18	N	Sep-04	Oct-14	10	15	42	Female	1	1	1	0.999979	>4
62	R291	18	N	Sep-04	Oct-14	10	14	31	Female	1	1	1	0.971466	>4
63	R291	18	N	Sep-04	Oct-14	10	13	23.5	Female	1	1	0.999976	0.195389	3 to 4
64	R291	18	N	Sep-04	Oct-14	10	12	18.5	Female	1	1	0.993991	0.008918	3 to 4
65	R291	18	N	Sep-04	Oct-14	10	11	6	Female	0.999093	0.044527	0.000171	2.38E-06	1 to 2
66	R291	18	N	Sep-04	Oct-14	10	10	4.5	Female	0.943622	0.005259	3.28E-05	8.85E-07	1 to 2

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
67	R291	18	N	Sep-04	Oct-14	10	9	3.5	Female	0.506614	0.001237	1.09E-05	4.58E-07	1 to 2
68	R291	18	N	Sep-04	Oct-14	10	8	2.5	Female	0.05926	0.00029	3.61E-06	2.37E-07	<1
69	R291	18	N	Sep-04	Oct-14	10	7	2.1	Male	0.020209	0.000162	2.32E-06	1.82E-07	<1
70	R291	17	M	Sep-04	Oct-14	10	18	46	Female	1	1	1	0.999999	>4
71	R291	17	M	Sep-04	Oct-14	10	17	42	Female	1	1	1	0.999979	>4
72	R291	17	M	Sep-04	Oct-14	10	16	26.5	Female	1	1	0.999999	0.636885	>4
73	R291	17	M	Sep-04	Oct-14	10	15	25.5	Female	1	1	0.999997	0.475717	3 to 4
74	R291	17	M	Sep-04	Oct-14	10	14	21	Female	1	1	0.999616	0.044657	3 to 4
75	R291	17	M	Sep-04	Oct-14	10	13	11	Female	1	0.985062	0.040695	6.42E-05	2 to 3
76	R291	17	M	Sep-04	Oct-14	10	12	8.5	Female	0.999999	0.636765	0.002688	1.24E-05	2 to 3
77	R291	17	M	Sep-04	Oct-14	10	11	5.5	Female	0.996348	0.022062	9.87E-05	1.71E-06	1 to 2
78	R291	17	M	Sep-04	Oct-14	10	10	4.5	Female	0.943622	0.005259	3.28E-05	8.85E-07	1 to 2
79	R291	17	M	Sep-04	Oct-14	10	9	4.2	Male	0.878713	0.003409	2.35E-05	7.26E-07	1 to 2
80	R291	17	M	Sep-04	Oct-14	10	8	3.5	Hermaphrodite	0.506614	0.001237	1.09E-05	4.58E-07	1 to 2
81	R291	17	M	Sep-04	Oct-14	10	7	3	Female	0.202758	0.000599	6.27E-06	3.29E-07	<1
82	A478	7841	N	Jul-11	Nov-14	3	17	19	Female	1	1	0.999998	0.953734	>4
83	A478	7841	N	Jul-11	Nov-14	3	16	15	Female	1	1	0.999826	0.5962	>4
84	A478	7841	N	Jul-11	Nov-14	3	15	23	Male	1	1	1	0.996537	>4
85	A478	7841	N	Jul-11	Nov-14	3	14	20	Male	1	1	0.999999	0.975519	>4
86	A478	7841	N	Jul-11	Nov-14	3	13	13	Male	1	0.999999	0.998425	0.283229	3 to 4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
87	A478	7841	N	Jul-11	Nov-14	3	12	11	Male	1	0.999991	0.985896	0.095638	3 to 4
88	A478	7841	N	Jul-11	Nov-14	3	11	4.5	Hermaphrodite	0.999964	0.897026	0.051221	0.001456	2 to 3
89	A478	7841	N	Jul-11	Nov-14	3	10	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
90	A478	7841	N	Jul-11	Nov-14	3	9	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
91	A478	7841	N	Jul-11	Nov-14	3	8	1.3	Female	0.784658	0.077376	0.001583	0.000177	1 to 2
92	A478	7841	N	Jul-11	Nov-14	3	7	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
93	A478	7841	N	Jul-11	Nov-14	3	6	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
94	A478	7841	N	Jul-11	Nov-14	3	5	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
95	A478	7841	N	Jul-11	Nov-14	3	4	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
96	A478	7841	N	Jul-11	Nov-14	3	3	0.3	Female	0.182698	0.019274	0.000526	9.15E-05	<1
97	A478	7839	M	Jul-11	Nov-14	3	18	23	Hermaphrodite	1	1	1	0.996537	>4
98	A478	7839	M	Jul-11	Nov-14	3	17	27	Male	1	1	1	0.999751	>4
99	A478	7839	M	Jul-11	Nov-14	3	16	21	Female	1	1	1	0.987184	>4
100	A478	7839	M	Jul-11	Nov-14	3	15	16	Female	1	1	0.999942	0.740532	>4
101	A478	7839	M	Jul-11	Nov-14	3	14	7.5	Female	1	0.998525	0.595901	0.010421	3 to 4
102	A478	7839	M	Jul-11	Nov-14	3	13	5.5	Female	0.999998	0.973804	0.139849	0.00281	2 to 3
103	A478	7839	M	Jul-11	Nov-14	3	12	3.5	Female	0.999409	0.671201	0.01761	0.000754	2 to 3
104	A478	7839	M	Jul-11	Nov-14	3	11	2	Female	0.96256	0.188031	0.003418	0.000281	1 to 2
105	A478	7839	M	Jul-11	Nov-14	3	10	2	Female	0.96256	0.188031	0.003418	0.000281	1 to 2
106	A478	7839	M	Jul-11	Nov-14	3	9	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
107	A478	7839	M	Jul-11	Nov-14	3	8	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
108	A478	7839	M	Jul-11	Nov-14	3	7	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
109	A478	7839	M	Jul-11	Nov-14	3	6	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
110	A478	7839	M	Jul-11	Nov-14	3	5	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
111	A478	7839	M	Jul-11	Nov-14	3	4	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
112	R291	15	N	Sep-04	Nov-14	10	18	50	Female	1	1	1	1	>4
113	R291	15	N	Sep-04	Nov-14	10	17	50	Female	1	1	1	1	>4
114	R291	15	N	Sep-04	Nov-14	10	16	60	Male	1	1	1	1	>4
115	R291	15	N	Sep-04	Nov-14	10	15	51	Male	1	1	1	1	>4
116	R291	15	N	Sep-04	Nov-14	10	14	49	Male	1	1	1	1	>4
117	R291	15	N	Sep-04	Nov-14	10	13	18	Female	1	0.999999	0.989618	0.00643	3 to 4
118	R291	15	N	Sep-04	Nov-14	10	12	11	Female	1	0.985062	0.040695	6.42E-05	2 to 3
119	R291	15	N	Sep-04	Nov-14	10	11	6	Female	0.999093	0.044527	0.000171	2.38E-06	1 to 2
120	R291	15	N	Sep-04	Nov-14	10	10	4.5	Female	0.943622	0.005259	3.28E-05	8.85E-07	1 to 2
121	R291	15	N	Sep-04	Nov-14	10	9	4	Female	0.80566	0.002553	1.89E-05	6.36E-07	1 to 2
122	R291	15	N	Sep-04	Nov-14	10	8	2.5	Female	0.05926	0.00029	3.61E-06	2.37E-07	<1
123	R291	15	N	Sep-04	Nov-14	10	7	2.5	Female	0.05926	0.00029	3.61E-06	2.37E-07	<1
124	R291	19	M	Sep-04	Nov-14	10	18	43	Female	1	1	1	0.999989	>4
125	R291	19	M	Sep-04	Nov-14	10	17	46	Female	1	1	1	0.999999	>4
126	R291	19	M	Sep-04	Nov-14	10	16	45	Female	1	1	1	0.999997	>4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
127	R291	19	M	Sep-04	Nov-14	10	15	26	Female	1	1	0.999998	0.557823	>4
128	R291	19	M	Sep-04	Nov-14	10	14	23	Female	1	1	0.999958	0.148691	3 to 4
129	R291	19	M	Sep-04	Nov-14	10	13	12.5	Female	1	0.998283	0.181475	0.000172	2 to 3
130	R291	19	M	Sep-04	Nov-14	10	12	11	Female	1	0.985062	0.040695	6.42E-05	2 to 3
131	R291	19	M	Sep-04	Nov-14	10	11	6	Female	0.999093	0.044527	0.000171	2.38E-06	1 to 2
132	R291	19	M	Sep-04	Nov-14	10	10	4.5	Female	0.943622	0.005259	3.28E-05	8.85E-07	1 to 2
133	R291	19	M	Sep-04	Nov-14	10	9	3.5	Female	0.506614	0.001237	1.09E-05	4.58E-07	1 to 2
134	R291	19	M	Sep-04	Nov-14	10	8	3	Female	0.202758	0.000599	6.27E-06	3.29E-07	<1
135	R291	19	M	Sep-04	Nov-14	10	7	3	Female	0.202758	0.000599	6.27E-06	3.29E-07	<1
136	A366	7553	N	Jul-11	Dec-14	3	17	25	Female	1	1	1	0.999071	>4
137	A366	7553	N	Jul-11	Dec-14	3	16	25	Female	1	1	1	0.999071	>4
138	A366	7553	N	Jul-11	Dec-14	3	15	23	Female	1	1	1	0.996537	>4
139	A366	7553	N	Jul-11	Dec-14	3	14	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
140	A366	7553	N	Jul-11	Dec-14	3	13	1.3	Female	0.784658	0.077376	0.001583	0.000177	1 to 2
141	A366	7553	N	Jul-11	Dec-14	3	12	4	Female	0.999854	0.808317	0.03017	0.001048	2 to 3
142	A366	7553	N	Jul-11	Dec-14	3	11	3	Female	0.997619	0.497032	0.010224	0.000542	1 to 2
143	A366	7553	N	Jul-11	Dec-14	3	10	1.7	Female	0.917549	0.130318	0.002458	0.00023	1 to 2
144	A366	7553	N	Jul-11	Dec-14	3	9	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
145	A366	7553	N	Jul-11	Dec-14	3	8	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
146	A366	7553	N	Jul-11	Dec-14	3	6	0.9	Male	0.544021	0.044833	0.001019	0.000136	1 to 2

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
147	A366	7553	N	Jul-11	Dec-14	3	5	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
148	A366	7551	M	Jul-11	Dec-14	3	15	21	Female	1	1	1	0.987184	>4
149	A366	7551	M	Jul-11	Dec-14	3	14	23	Female	1	1	1	0.996537	>4
150	A366	7551	M	Jul-11	Dec-14	3	13	14.5	Female	1	1	0.999698	0.515024	>4
151	A366	7551	M	Jul-11	Dec-14	3	12	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
152	A366	7551	M	Jul-11	Dec-14	3	11	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
153	A366	7551	M	Jul-11	Dec-14	3	10	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
154	A366	7551	M	Jul-11	Dec-14	3	9	0.9	Female	0.544021	0.044833	0.001019	0.000136	1 to 2
155	A366	7551	M	Jul-11	Dec-14	3	8	0.9	Female	0.544021	0.044833	0.001019	0.000136	1 to 2
156	A366	7551	M	Jul-11	Dec-14	3	7	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
157	A366	7551	M	Jul-11	Dec-14	3	6	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
158	A366	7551	M	Jul-11	Dec-14	3	5	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
159	A366	7551	M	Jul-11	Dec-14	3	4	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
160	A366	7551	M	Jul-11	Dec-14	3	3	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
161	A366	7551	M	Jul-11	Dec-14	3	2	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
162	A366	7551	M	Jul-11	Dec-14	3	1	0.3	Female	0.182698	0.019274	0.000526	9.15E-05	<1
163	A366	7536	N	Jul-11	Feb-15	3	17	13.5	Female	1	1	0.999092	0.354581	3 to 4
164	A366	7536	N	Jul-11	Feb-15	3	16	15.5	Female	1	1	0.9999	0.67243	>4
165	A366	7536	N	Jul-11	Feb-15	3	15	11.5	Female	1	0.999996	0.991824	0.128183	3 to 4
166	A366	7536	N	Jul-11	Feb-15	3	14	11	Female	1	0.999991	0.985896	0.095638	3 to 4

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
167	A366	7536	N	Jul-11	Feb-15	3	13	6	Female	0.999999	0.987145	0.220062	0.003903	2 to 3
168	A366	7536	N	Jul-11	Feb-15	3	12	3.5	Female	0.999409	0.671201	0.01761	0.000754	2 to 3
169	A366	7536	N	Jul-11	Feb-15	3	11	2.5	Female	0.990458	0.323581	0.005917	0.00039	1 to 2
170	A366	7536	N	Jul-11	Feb-15	3	10	2	Female	0.96256	0.188031	0.003418	0.000281	1 to 2
171	A366	7536	N	Jul-11	Feb-15	3	9	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
172	A366	7536	N	Jul-11	Feb-15	3	8	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
173	A366	7536	N	Jul-11	Feb-15	3	7	0.9	Female	0.544021	0.044833	0.001019	0.000136	1 to 2
174	A366	7536	N	Jul-11	Feb-15	3	6	0.8	Female	0.474378	0.039014	0.000913	0.000127	<1
175	A366	7536	N	Jul-11	Feb-15	3	5	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
176	A366	7554	M	Jul-11	Feb-15	3	15	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
177	A366	7554	M	Jul-11	Feb-15	3	14	6.5	Female	1	0.993736	0.328701	0.005418	2 to 3
178	A366	7554	M	Jul-11	Feb-15	3	12	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
179	A366	7554	M	Jul-11	Feb-15	3	11	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
180	A366	7554	M	Jul-11	Feb-15	3	10	2	Female	0.96256	0.188031	0.003418	0.000281	1 to 2
181	A366	7554	M	Jul-11	Feb-15	3	9	1.2	Female	0.733783	0.067632	0.001418	0.000166	1 to 2
182	A366	7554	M	Jul-11	Feb-15	3	8	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
183	A366	7554	M	Jul-11	Feb-15	3	7	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
184	A366	7554	M	Jul-11	Feb-15	3	6	0.7	Female	0.405717	0.033923	0.000817	0.000119	<1
185	A366	7554	M	Jul-11	Feb-15	3	5	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
186	A366	7554	M	Jul-11	Feb-15	3	4	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
187	A366	7554	M	Jul-11	Feb-15	3	3	0.5	Female	0.280913	0.025597	0.000656	0.000104	<1
188	A366	7554	M	Jul-11	Feb-15	3	2	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
189	A366	7554	M	Jul-11	Feb-15	3	1	0.3	Female	0.182698	0.019274	0.000526	9.15E-05	<1
190	A366	7554	M	Jul-11	Feb-15	3	0	0.3	Female	0.182698	0.019274	0.000526	9.15E-05	<1
191	A366	7558	M	Jul-11	Feb-15	3	16	27	Female	1	1	1	0.999751	>4
192	A366	7558	M	Jul-11	Feb-15	3	15	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
193	A366	7558	M	Jul-11	Feb-15	3	14	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
194	A366	7558	M	Jul-11	Feb-15	3	13	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
195	A366	7558	M	Jul-11	Feb-15	3	12	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
196	A366	7558	M	Jul-11	Feb-15	3	11	6	Female	0.999999	0.987145	0.220062	0.003903	2 to 3
197	A366	7558	M	Jul-11	Feb-15	3	10	2.5	Female	0.990458	0.323581	0.005917	0.00039	1 to 2
198	A366	7558	M	Jul-11	Feb-15	3	8	1.5	Female	0.864275	0.100802	0.001973	0.000202	1 to 2
199	A366	7558	M	Jul-11	Feb-15	3	7	0.9	Female	0.544021	0.044833	0.001019	0.000136	1 to 2
200	A366	7558	M	Jul-11	Feb-15	3	6	1.3	Female	0.784658	0.077376	0.001583	0.000177	1 to 2
201	A366	7558	M	Jul-11	Feb-15	3	5	0.8	Female	0.474378	0.039014	0.000913	0.000127	<1
202	A366	7558	M	Jul-11	Feb-15	3	4	1	Female	0.611984	0.051474	0.001138	0.000145	1 to 2
203	A366	7558	M	Jul-11	Feb-15	3	3	0.6	Female	0.340556	0.029477	0.000732	0.000112	<1
204	A366	7558	M	Jul-11	Feb-15	3	2	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
205	A366	7558	M	Jul-11	Feb-15	3	1	0.4	Female	0.228102	0.022217	0.000587	9.78E-05	<1
206	A366	7558	M	Jul-11	Feb-15	3	0	0.3	Female	0.182698	0.019274	0.000526	9.15E-05	<1

Sl No	Clone	Palm	Phenotype	Field planting date	Sampling date	Age at sampling	Frond No	Length	Sex	1 probability	2 probability	3 probability	4 probability	Predicted stage
207	R291	23	M	Sep-04	Nov-18	14	25	44	Female	1	1	1	0.999615	>4
208	R291	23	M	Sep-04	Nov-18	14	14	40.15	Female	1	1	1	0.995158	>4
209	R291	23	M	Sep-04	Nov-18	14	11	14.5	Female	1	0.993533	0.028357	9.35E-06	2 to 3
210	R291	23	M	Sep-04	Nov-18	14	13	34	Female	1	1	1	0.781133	>4
211	R291	23	M	Sep-04	Nov-18	14	12	19	Female	1	0.99999	0.806442	0.000182	3 to 4
212	R291	23	M	Sep-04	Nov-18	14	10	5.5	Female	0.798375	0.000327	1.43E-06	2.48E-08	1 to 2
213	R291	23	M	Sep-04	Nov-18	14	9	6	Female	0.941131	0.000676	2.49E-06	3.45E-08	1 to 2
214	R291	16	N	Sep-04	Nov-18	14	25	47	Female	1	1	1	0.999947	>4
215	R291	16	N	Sep-04	Nov-18	14	15	30	Female	1	1	0.999999	0.203585	3 to 4
216	R291	16	N	Sep-04	Nov-18	14	14	24	Female	1	1	0.999032	0.004876	3 to 4
217	R291	16	N	Sep-04	Nov-18	14	13	18	Female	1	0.999959	0.580438	9.39E-05	3 to 4
218	R291	16	N	Sep-04	Nov-18	14	12	13.3	Female	1	0.964201	0.007713	4.24E-06	2 to 3
219	R291	16	N	Sep-04	Nov-18	14	11	8.4	Female	0.999923	0.021532	3.5E-05	1.68E-07	1 to 2
220	R291	16	N	Sep-04	Nov-18	14	10	5.8	Male	0.901458	0.000506	1.99E-06	3.03E-08	1 to 2
221	R291	16	N	Sep-04	Nov-18	14	9	4.5	Male	0.19544178	7.67198E-05	4.75495E-07	1.28418E-08	<1

APPENDIX 7

Census Data

Data on the sex of inflorescence at leaf stages F14 to F19 was collected via census from genotyped and phenotyped palms prior to sampling as tabulated below. The phenotype of the palm is indicated by letters M and N for mantled and normal respectively. The frond number indicates the sequential order of the subtending leaf from the spear. The sex of inflorescence is indicated by letters F, S and U for female, male and unknown respectively. Census focused on normal palms as this data was used to decide on sampling times during which there are more female inflorescences available in normal palms. This was done to ensure the availability of comparable samples as mantled palms were found to enter the male phase less frequently (discussed in chapter 9). All palms were part of the same replicate (A) except those marked * which were part of replicate B. Within a replicate, the macro and micro environments were considered identical.

Date of census	Clone	Palm	Phenotype	Sex of Inflorescence at respective Frond Numbers						Male	Female	Hermaphrodite	Total
				F14	F15	F16	F17	F18	F19				
11/8/2014	A229	67	M	U	U	U	F	F	S	1	2	0	3
11/8/2014	A229	68	N	U	U	U	S	S	F	2	1	0	3
11/8/2014	A229	70	N	S	S	S	S	S	F	5	1	0	6
11/8/2014	A229	71	N	F	F	F	F	F	F	0	6	0	6
11/8/2014	A229	72	N	U	U	S	S	F	S	3	1	0	4
11/8/2014	A229	73	N	U	S	F	F	S	S	3	2	0	5
11/8/2014	A229	77	M	F	F	F	F	F	F	0	6	0	6
11/8/2014	R291	15	N	U	F	F	F	F	F	0	5	0	5
11/8/2014	R291	16	N	S	S	S	S	F	F	4	2	0	6
11/8/2014	R291	18	N	S	S	S	F	F	F	3	3	0	6
11/8/2014	R291	01*	N	U	F	F	F	F	F	0	5	0	5
11/8/2014	R291	02*	N	U	U	S	S	F	S	3	1	0	4
11/8/2014	R291	03*	N	F	F	F	F	F	S	1	5	0	6
11/8/2014	R291	05*	N	U	U	S	F	F	F	1	3	0	4
11/8/2014	R295	24	N	U	S	S	F	F	F	2	3	0	5
11/8/2014	R295	01*	N	U	F	F	F	F	F	0	5	0	5
11/8/2014	R295	02*	N	S	S	S	S	S	F	5	1	0	6
11/8/2014	R295	03*	N	U	U	F	F	F	F	0	4	0	4
11/8/2014	R295	05*	N	F	F	S	S	F	F	2	4	0	6
22/8/2014	R291	15	N	U	U	F	F	F	F	0	4	0	4
22/8/2014	R295	24	N	U	U	U	U	U	U	0	0	0	0
22/8/2014	R295	21	M	F	F	F	F	F	F	0	6	0	6
22/8/2014	R291	17	M	U	F	?	F	F	F	0	4	0	4

GLOSSARY

Abiotic stress: The negative impact of non-living factors on the living organisms in a specific environment.

Acetylation: The chemical addition of an acetyl functional group into a chemical compound.

Acropetal: Growth or development upwards from the base or point of attachment.

Allele: A variant form of a gene.

Allogamous: Exhibiting allogamy, that is reproduction by cross-fertilization.

Amplicon: A piece of DNA or RNA that is the product of amplification events.

Androecium: The male reproductive whorl of a flower, consisting of one or more stamens.

Aneuploidy: The presence of an abnormal number of chromosomes in a cell.

Anther: The part of the stamen where pollen is produced.

Anthesis: The flowering period of a plant, from the opening of the flower bud.

Bract: A modified leaf with a flower or inflorescence in its axil.

Calyx: The sepals of a flower, typically forming a whorl that encloses the petals and forms a protective layer around a flower in bud.

Carpel: The female reproductive organ of a flower, consisting of an ovary, a stigma, and usually a style.

Centrifugation: Technique that helps to separate mixtures by applying centrifugal force.

Clone: An organism (ramet) or a group of organisms produced asexually from one ancestor (ortet), to which they are genetically identical.

Corolla: The petals of a flower, typically forming a whorl within the sepals and enclosing the reproductive organs.

Cryopreservation: A process that preserves biological structures by cooling them to very low temperatures.

Dehiscence: The splitting or bursting open of anther to release pollen grains.

Dioecious: Exhibiting dioecy or having male and female reproductive organs in separate individuals.

Drupe: A fleshy fruit with thin skin and a central stone containing the seed.

Dominance: One allele of a gene masking or overriding the effect of others.

Ecotype: A distinct form of a species occupying a particular habitat.

Elution: The chromatographic process of using a solvent to extract an adsorbed substance from a solid adsorbing medium

Endocarp: The innermost layer of the pericarp which surrounds a seed in a fruit.

Endosperm: Tissue that surrounds and nourishes the embryo in the seeds of angiosperms.

Epigenetic: Relating to changes to the gene expression without changes to the underlying DNA sequence.

Epigenome: The complete set of chemical modifications to DNA and histone proteins that regulate the expression of genes within the genome.

Ester: Chemical compound derived from an acid and an alcohol.

Esterase: A hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction called hydrolysis.

Exocarp: The outer layer of the pericarp of a fruit.

Explant: A part of the plant by which a whole plant can be produced through plant tissue culture technique

Fertility: The ability to procreate successfully.

Fertilization ability: Ability of pollen to fertilize female gametophyte.

Fingerprinting: (In DNA or Genetic Fingerprinting) A method used to identify an individual by looking at unique patterns in their DNA.

Flaccid: Lacking the normal firmness or plumpness.

Genome: The complete set of genes present in a cell or organism.

Germinability: The ability of a pollen grain to develop a pollen tube.

Germplasm: Germ cells and their precursors serving as the bearers of heredity.

Gynoecium: The female reproductive whorl of a flower, consisting of one or more carpels.

Heliophytic: A plant thriving in or tolerating full sunlight.

Hermaphrodite: The condition of having both male and female reproductive organs.

Heterogeneity: The quality or state of being diverse in character or content.

Homeosis: Transformation of one organ into another.

Homology: The state of having the same or similar relation, relative position, or structure.

Hydrolysis: A chemical reaction involving the interaction of chemicals with water, leading to the decomposition of both.

Hypomethylation: The loss of the methyl group in the 5-methylcytosine nucleotide

Indeterminate: A shoot not having all the axes terminating in a flower bud and so potentially of indefinite length.

Longevity: The duration for which pollen is viable and can germinate after anther dehiscence.

Meiosis: A process where a single cell divides twice to produce four cells containing half the original amount of genetic information.

Mendelian inheritance: An inheritance pattern that follows the laws of segregation and independent assortment.

Meristem: (In plants) A region of unspecialised cells capable of cell division.

Mesocarp: The pulpy middle layer of the pericarp of a fruit, between the endocarp and the exocarp.

Mixoploidy: The situation where two cell lines, one diploid and one polyploid, coexist within the same organism.

Molecular Markers: Variable DNA sequences that have been mapped to a position on the chromosome.

Monocotolydonous: A flowering plant having a single cotyledon in the seed.

Monoecious: Exhibiting monoecy or having both male and female reproductive organs in the same individual.

Monofactorial: (With reference to a trait) governed by just one gene with two alleles.

Monomorphic Marker: A molecular marker that shows no variation in morphology amongst the individual studied.

Non-polar: A Molecule that has no separation of charge

Ortet: The original plant or the source of an explant from which the members of a clone have descended.

Orthotropous: Having the ovule straight and upright with the micropyle at the apex.

Ovary: Enlarged basal portion of the pistil. Upon fertilization ovules within the ovary develop into seeds and the ovary itself will mature into a fruit.

Ovule: Plant structure that develops into a seed when fertilized.

Pedicellate: (In plant structure) Having a stalk or pedicel.

Peduncle: The stalk of a flower or an inflorescence.

Peduncular bract: A bract on the peduncle located on the main axis of the inflorescence between the prophyll and the first bract of the rachis.

Perianth: The non-reproductive whorls of the flower that envelope the sexual organs, consisting of the calyx (sepals) and the corolla (petals) or tepals.

Petal: Each of the segments of the corolla of a flower, which are modified leaves and are typically coloured.

Phenotype: An individual's observable traits.

Phosphorylation: The chemical addition of a phosphoryl group (PO^{3-}) to an organic molecule.

Photoperiod: The recurring cycle of uninterrupted light and dark periods a plant is exposed to.

Pioneer-species: Species that arrive first in a newly created environment.

Plasticity: (With reference to development) Changes in the developmental trajectory adopted by an organism, influenced by environmental factors or gene–environment interactions.

Pleonanthy: Flowering behaviour involving the production of inflorescences continually and indeterminately throughout the vegetative extension.

Pollen abortion: Premature termination of the development of pollen.

Pollen Load: The mean number of pollen grains produced by a flower.

Pollen mother cell: A cell derived from the hypodermis of the pollen sac that gives rise to 4 pollen grains through meiosis.

Polymorphic: (In genetic variation) Occurring in several different forms.

Pollen sac: The structure that contains the microspore mother cells and produces pollen. In angiosperms, there are usually 4 in each anther.

Polyphenols: Secondary metabolites produced by plants.

Polyploidy: The heritable condition of possessing more than two complete sets of chromosomes.

Primordium (Plural: primordia): An organ or tissue in its earliest recognizable stage of development.

Prophyll: A leaf-like structure subtending a single flower or inflorescence.

Pseudocarpels: Supernumerary carpels formed by the homeotic transformation of stamens or staminodes.

Rachilla or Spikelet: Secondary rachis or branches of a rachis.

Rachis: A stem of a plant bearing flower stalks at short intervals.

Ramet: A physiologically distinct individual of a clone.

Restriction endonuclease or Restriction enzyme: A protein produced by bacteria that cleaves DNA at specific sites along the molecule.

Retrotransposon: A chromosomal segment that can copy and paste itself into different locations in the genome using an RNA transposition intermediate.

Sepal: Each of the parts of the calyx of a flower, enclosing the petals and is typically green and leaflike.

Sessile: (In plant structure) Attached directly by its base without a stalk or peduncle.

Sex ratio: The proportion of female inflorescences divided by the total inflorescences produced in a specified group of plants.

Somaclonal Variation: Genetic or epigenetic changes that arise from tissue culture between clonal regenerants.

Somatic embryogenesis: The developmental process where a plant somatic cell produces embryos in vitro.

Spadix: An inflorescence composed of minute flowers closely arranged around a fleshy axis and typically enclosed in a spathe.

Spathe: A large sheathing bract enclosing the inflorescence of certain plants, especially the spadix of palms.

Spear leaf: Unopened leaf that appears as a spike at the crown of the palm.

Stainability: The capacity of cells and cell parts to stain specifically and consistently with particular dyes and stains.

Stamens: Male reproductive organs of the flower that produce pollen.

Staminodes: Rudimentary, sterile or abortive stamen.

Sterility: Unable to produce viable offspring.

Stigma: Specially adapted portion of the pistil modified for the reception of pollen.

Subtending leaf: The leaf that occurs beneath or close to an inflorescence.

Supernatant: The liquid lying above a solid residue after precipitation, centrifugation, or other processes.

Syncarpous: (In a flower, fruit or ovary) Having fused or united carpels.

Tepal: A segment of the outer whorl in a flower, where there are no morphological differences between petals and sepals.

Testa: Seed coat that protects the embryo against adverse environmental conditions.

Transcription: The process of transcribing RNA (transcripts), using a DNA template.

Transcriptome: The total of all the messenger RNA molecules expressed from the genes of an organism.

Transposon or Transposable elements (TEs): A chromosomal segment that can move from one location on the genome to another.

Trichotomosulcate: Having three distal sulci or furrows or slits.

Trimerous: Having parts arranged in groups of three.

Ubiquitylation or ubiquitination: The chemical addition of ubiquitin, a small protein found in eukaryotic organisms, to another targeted protein.

Vernalization: The induction of a plant's flowering process by exposure to the prolonged cold of winter, or by an artificial equivalent.

Viability: The capacity of pollen to live, grow, germinate, or develop.

Vigour: The potential level of activity and performance of the pollen during germination and pollen tube emergence.

Vitality: The ability of the pollen to perform its function of delivering male gametes to the embryo sac.

Vortexing: Mixing laboratory samples using a vortex.

This glossary is prepared using definitions from online sources.