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RNA-Seq analysis reveals influence of sugar level and photoperiod on seasonality in oil palm (*Elaeis guineensis* Jacq.) sex-specific inflorescence emergence

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ABSTRACT

We induced male inflorescence formation on 162 oil palm trees of 14 years old by complete defoliation. Inflorescence emergence data was analysed within a five-year period. Total RNA was extracted from un-emerged inflorescences of both completely defoliated and non-defoliated trees for gene expression studies. The aim was to investigate the effects of voluntary time-specific inflorescence induction on the seasonal trend of inflorescence emergence; including the molecular mechanisms regulating inflorescence emergence in oil palm. Male inflorescence emergence increased by 104.2% after complete defoliation stress treatment. The time between induction and emergence of inflorescence was 17.83 months. There was an increase of 58.8% in male inflorescence emergence during the wet season as compared to the dry season although equal numbers of trees were treated for each season. Male inflorescence emergence was seasonal irrespective of the time or season of inflorescence induction, indicating that sex specific inflorescence emergence in oil palm is pre-programmed and synchronised, depending on seasonal cues. Response to carbohydrate status, light and temperature were among the highly enriched functional clusters obtained from 1,214 differentially expressed genes (DEG). Knowing the factors controlling inflorescence emergence, crop production estimation and breeding strategies can efficiently be designed by agronomists and breeders.

1. INTRODUCTION

Unlike in annuals such as Arabidopsis in which flower induction and emergence apparently concur in time, the interval between inflorescence induction and emergence in the perennial tree crops is rather distinct 510. It may take several months to years between flower induction and flower emergence in major perennial crops. For example, the flowering process in grapevine takes 2 years from vegetative phase transition of meristem, through meristem development to flower emergence 12 13. Similarly, Adam *et al.* 1, Cros *et al.* 16 and Legros *et al.* 27 have estimated that it takes between 18 to 22 months from inflorescence induction to inflorescence emergence in oil palm.

Thus the environmental factors and molecular mechanisms regulating flower induction and flower emergence may be confounding in short life-cycle annuals while these factors may be distinct in perennials because of the longer time interval between induction and emergence.

Flowering time has been well studied in Arabidopsis in which five pathways are thought to regulate flowering time. Several reports indicate that photoperiod 39 vernalisation 25, autonomous 33, ageing 20 and GA 34 pathways regulate flowering time in Arabidopsis.

Flower emergence is also regulated by discrete environmental cues related to photoperiod, temperature, water and also, genetic factors and carbohydrate 3943. The oil palm is monoecious, that is the male and female flowers occur separately on the same plant. It has been shown in a previous study 4 that the amounts of sugar levels determine inflorescence sex induction in oil palm.

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Oil palm trunks contain considerable energy reserves that are used to supply assimilate to sinks during periods of high-energy demand or during reduced photosynthetic activities. The total extractable carbohydrate in the trunk of an eight-year-old palm amounted to 37 Kg. The genetic basis of flowering emergence time especially in annuals, has gained sufficient attention because of its agronomic importance 34, 39,43. In contrast, molecular mechanisms controlling flowering in perennials have not been given sufficient interest when compared to the massive information available on flowering in annuals 24. RNA-seq may be used for the detection of cellular pathway alterations and differential gene expression during abiotic stress 29.

Knowing the factors that control inflorescence emergence and understanding the physiological and genetic basis can help tree crop plantation managers to accurately estimate the trend of future crop harvests especially in this era of global climate change 11, 8, 40. Breeders would develop plants with specific tolerance to particular environmental factors using such knowledge. The elucidation of genes underlying plants' responses to environmental changes can give important information about their ability to respond to climate instability.

Production estimation is important in plantation agriculture especially to plan and efficiently use limited available resources. Plantation managers are expected to make yearly estimates through forecasts. It should be interesting to know whether abiotic stress caused by an unexpected change in climatic conditions, would affect seasonal production trend 17 so as to effectively plan annual work program and mitigate crop loss. Managers are faced with the inability to exactly determine how an abrupt change in today's weather conditions would affect production trend. Today, plantation managers do not have total control over these phenomena especially as they are constantly faced with unstable weather conditions.

Here, we use both phenotypic and genomic data to analyse inflorescence emergence pattern and the physiological factors that regulate flowering time in a monoecious perennial tree crop, the oil palm. The purposes of this research were to examine (i) the effects of voluntary inflorescence induction by complete defoliation on the seasonal trend of inflorescence emergence and (ii) the physiological factors related to inflorescence emergence in oil palm by transcriptome analysis. We hypothesized that the number of months between inflorescence induction and inflorescence emergence in oil palm is fixed; that inflorescence emergence occurs after a fixed number of months regardless of the season in which induction treatment was done and regardless of the prevailing environmental conditions during emergence stage; that DEGs from total RNA extracted from mature un-emerged inflorescence of completely defoliated and non-defoliated tree replicates would provide the necessary information on the molecular mechanisms related to inflorescence emergence time. Substantial information on the seasonality nature of oil palm inflorescence emergence was obtained based on physiological and climatic analyses. The findings were later elucidated using molecular methods so as to provide an understanding of the mechanisms regulating inflorescence emergence in oil palm.

2. MATERIALS AND METHODS

2.1 Plant material and research sites

The oil palm that was used in this study is the shell-less type known as *Pisifera*, planted in 1993 at the Oil Palm Research Centre of the Institute of Agricultural Research for Development at La Dibamba, Cameroon (3.948848°N, 9.762726°E). There are two distinct seasons at La Dibamba: the dry season and the wet season. The wet season spans from 15th of March to 15th of October while the dry season spans from 16th of October to 14th of March.

Plant tissues were extracted from six trees on un-emerged inflorescence located at leaf axil N^{o} + 5 for molecular studies and soluble sugar analysis. Three trees (3 biological replicates) were completely defoliated (DD) while another set of 3 trees was not defoliated (ND). The trees were felled 45 days after defoliation (DAD) and leaf petioles were successively removed to extract the un-emerged inflorescences located between the petiole and the trunk (leaf axil).

It has been shown in a preceding study that the *Pisifera* tree takes forty-five days to respond to complete defoliation stress 3. Total soluble sugar measurement and genomic analysis were done in the laboratory of Macrogen Next Generation Sequencing Division, Seoul South Korea.

2.2 Inflorescence induction treatment

Inflorescence induction was done through complete defoliation of the tree as described in Durand-Gasselin *et al.* 18 and Ajambang *et al.* 3. Weekly observations were carried out on the trees to record the total number of inflorescences produced. Inflorescences were harvested at the stage of pollen maturity or anthesis and were taken to the laboratory for pollen collection and quantification. Inflorescence emergence data was collected from the 10th month through to the 24th month following induction. Total soluble sugar was measured as reported in Ajambang *et al.* 3.

2.3Phenotypic and genomic data analysis

Data was analysed with SAS version 9.3 (SAS Institute Inc, Cary NC). A paired *t*- test ($P \leq 0.05$) was used to compare the effects on the same individual trees before and after treatment while a two – sample *t* – test was used to compare response from averages of two different sets of data. RNA isolation, sequencing and data analysis were done as explained in Ajambang *et al.* 4.

3. RESULTS

3.1Male inflorescence production response to complete defoliation

Total number of inflorescences and quantity of pollen were recorded on the same trees before and after complete defoliation. Table 1 shows values for male inflorescence and

pollen production before and after complete defoliation. An increase of 104.2% in total number of male inflorescences was observed after complete defoliation treatment. A paired *t* – test showed that there was a significant effect on total male inflorescence production after complete defoliation (x = 7.1, SD = 4.09) than was observed before complete defoliation (x = 3.7, SD = 3.47), t (41) = 5.99, P< 0.000.

Table 1: Number of male inflorescence and quantity of pollen produced before and after defoliation.

Observed data	# FBD	# FAD	QPBD	QPAD
Total	143.0	292.0	1898.0	5712.0
Average/tree	3.7	7.1	46.3	139.3
% Increase	104		201	
T-value	**5.99****		**3.82****	

Legend: #FBD = Total number of male inflorescences produced 2 years preceding complete defoliation, #FAD = Total number of inflorescences produced 2 years after complete defoliation. Two years was retained as data collection limit because the treatment has it maximum effect between 15 and 24 months. QPBD = Total quantity of pollen produced before complete defoliation, QPAD = Total quantity of pollen produced after defoliation. ** (Significant difference at $P \leq 0.05$).

Total soluble sugar was analysed on developing inflorescences and the results are represented on the graph in Figure 1. There was a decrease of 21% in soluble sugar content between the non defoliated plants and defoliated plants at 45 DAD.

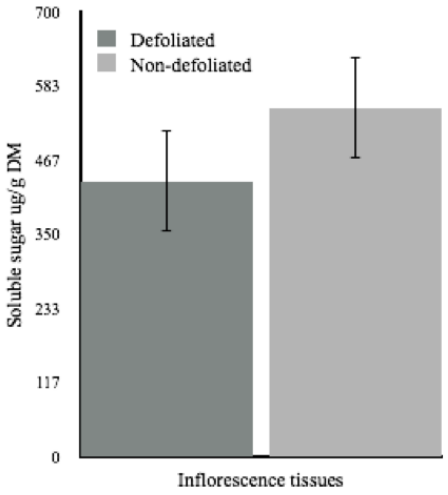

Fig. 1: Total soluble sugar from emerging inflorescences of defoliated and non defoliated plants

3.2Time to first male inflorescence production after complete defoliation

We recorded inflorescence production on each defoliated tree for a 24-month period: that is two dry seasons and two wet seasons. An analysis of the distribution of first inflorescence production on the individual trees showed that 25% of the first inflorescences matured 15.54 months after complete defoliation. Figure 2 shows that the earliest inflorescences were produced 10.77 months after complete defoliation while 50% of the first inflorescences were produced at 18.23 months. The maximum time recorded between treatment and male inflorescence production was 23.73 months while the mean number of months to inflorescence production was 17.83 months.

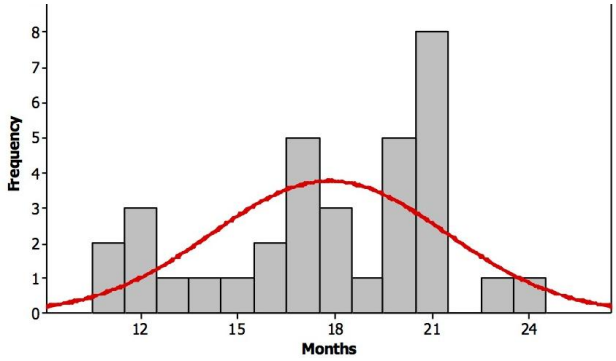

Fig. 2. Distribution of male inflorescence production in months after complete defoliation

3.3 Difference in male inflorescence Production between the wet season and the dry season

As part of our hypotheses, we sought to find out if there was a significant difference in inflorescence production between the wet season and dry season although equal numbers of trees were defoliated in the wet and dry seasons. A *t*-test was performed on the averages of male inflorescence production from the two seasons and the results show that there was a significant difference in inflorescence production between the wet season and the dry season. There was a higher production of inflorescence (58.8%) during the wet season irrespective of the time and season of inflorescence induction. Production in the wet season was significantly higher than during the dry season no matter the season in which defoliation was done (*t*-value = 2.96**, *P*-value = 0.0071) at *P*=0.05.

Table 2: *t*-test for number of inflorescence induction and production between the wet and dry season

		Production season			
Defoliation season	**Variables**	**Wet season**	**Dry season**	**Row totals**	***t*-value**
	Wet season	79	15	**94**	**1.74****
	Dry season	130	28	**158**	
	Column totals	**209**	**43**	**252**	
	***t*-test**	**2.96****			

Legend: Column totals represent the total number of male inflorescences that were produced and harvested in each season. The row totals represent total number of male inflorescences that were produced when defoliation treatment was done in each of the seasons. The *t*-test value for the column totals compares inflorescences produced in each season. The *t*-value for row totals compares the efficiency of male inflorescence induction when defoliation was done in each season. ** denotes that the *t*-test value was significantly at P = 0.05.

The practical implication of complete defoliation in oil palm is to induce male inflorescences for the supply of pollen during artificial seed production and breeding. In this study we also conducted tests to find out which of the seasons is better for complete defoliation in terms of total number of male inflorescences emerged. Results (Table 2) show that more inflorescences were produced when complete defoliation was done

in the dry season (*t*-value = 1.74, *P*-value = 0.044) than was done in the wet season although the same numbers of trees were defoliated in each season.

3.4Transcriptome analysis and DEG on mature emerging inflorescence

A total of 96, 180 genes were successfully mapped to the oil palm genome. 60,702 genes were retained after filtering and normalization. These genes were aligned to the Arabidopsis genome database. Out of the 60,702 genes, 47,832 genes were co-regulated in both stress and control tissues. 28,636 were up regulated and 19,149 genes were down regulated in both the control and stress tissues. 6,427 genes were up regulated in control samples but were down regulated in stressed samples while 6,488 genes were up regulated in stress samples but down regulated in control samples. Figure 3 shows a Venn diagram of DEGs in control and stressed tissues.

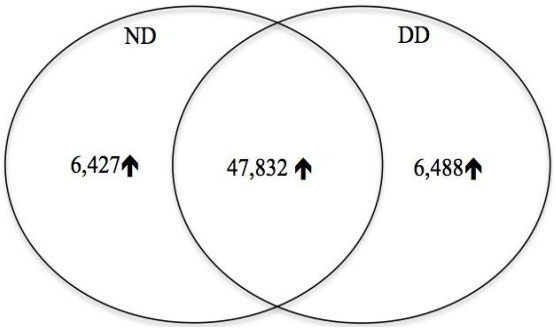

Fig. 3: Venn diagram of up regulated DEGs on non defoliated (ND) and defoliated (DD) samples

The volcano plot presented in Figure 4 gives the distribution of comparative gene expression between the defoliated and non-defoliated samples. The farther the distance from the fold change limits, the higher the fold change. Genes positioned on the left limit are down regulated while those on the right are up regulated.

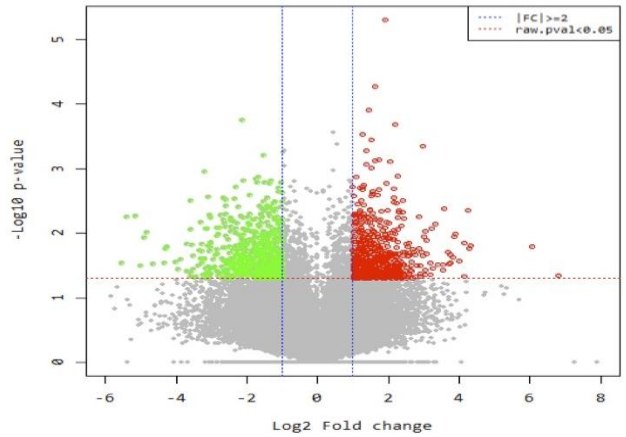

Fig. 4: Volcano plot for DEGs between non-defoliated and defoliated samples.

The y-axis represents the level of significance of the expression change between samples measured on –log10 p-value (pval = 0.05), while the x-axis represents the fold change of DEGs. DEGs located above the red line are significant based on the p-value while those below are not significant. The DEGs located between the two blue lines are non DEGs based $|FC| \geq 2$.

3.5Seasonality in inflorescence emergence

Inflorescence emergence is the production of inflorescences on the tree while inflorescence induction or initiation is the transition from vegetative to reproductive phase of the leaf axil meristem. The interval separating inflorescence induction and emergence in oil palm tree was calculated in this study as 17.8 months while it was estimated to be between 18 to 24 months by Durand-Gasselin *et al.* 18 and Legros *et al.* 27. We sought to know if the trend of inflorescence emergence followed the same trend as the monthly inflorescence induction. That is, if inflorescences sequentially appeared according to when their respective trees were defoliated. Equal numbers of trees were defoliated each month. Figure 5 presents the average monthly number of inflorescences emerged based on the season of defoliation treatment.

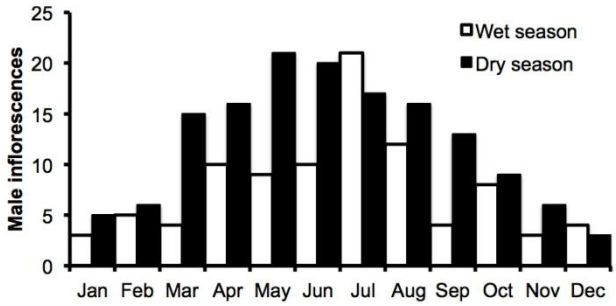

Fig. 5: Average monthly emergence of male inflorescences based on the season of defoliation treatment.

Every month has two bars; the white coloured bar represents the average number of monthly inflorescences emerged when defoliation treatment was done in the wet season and the black coloured bar represents average number of monthly inflorescences emerged when defoliation was done in the dry season. Flower emergence increased from March to October, which also coincides with the wet season.

4. DISCUSSIONS

It should be recalled here that at La Dibamba the rainy season runs from the 15th of March to the 15th of October while the dry season runs from the 16th of October to the 14th of March. It can be observed (Fig. 5) that the majority of inflorescences were produced between March and October irrespective of the season of induction. According to the hypothesis, it was expected that the number of inflorescences emerged would be the same for each season because the number of trees defoliated was equal for both seasons. This study shows that there is preferential emergence of inflorescence based on the season. This is an indication that we cannot determine the date on which an inflorescence will emerge although we voluntarily induced the inflorescence some 18 to 24

months ago. Hence, inflorescence emergence is not dependent on the date of induction, but is dependent on some environmental factors prevailing at the time the inflorescence is ready to be emerged out of the leaf axil.

These environmental factors are often known as seasonal cues, which include weather parameters such as photoperiod, temperature, humidity and soil water availability 26143, 4237. It may seem that the wet season has some particular environmental factors that are involved in cross talk with carbohydrate balance culminating in the emergence of inflorescences. According to Andres and Coupland 6 Adam *et al.* 1 and Wong *et al.* 42 the most prominent environmental factors that influence flower emergence in plants are day length, carbohydrate supply and soil water availability. The oil palm is a tropical plant and should be neutral to day length because of its insignificance in the tropics.

Corley 15 and Cros *et al.* 16 observed high male inflorescence emergence during the wet season in oil palm commercial plantations. Researchers have observed that the emergence of flowers on certain crops followed particular seasonal cues and not dependent on a fixed period after flower initiation. It has been reported that soil water deficit caused delayed inflorescence emergence in maize and sorghum 19 while it anticipated inflorescence emergence in barley and wheat 31. It seems that there is a delay of inflorescence emergence, for inflorescences that are ready to be emerged but have to wait until the appropriate environmental conditions prevail. When these seasonal cues prevail, the entire ready-to be-emerged, cued inflorescences come out synchronously from the leaf axils. Likewise, there should be anticipation of inflorescence emergence when favourable environmental conditions are prevailing, especially on inflorescences close to emergence stage.

When seeking for planting material, tree crop plantation managers usually demand for genetically uniform seed lots. This is because use of uniform planting material enables efficient managenent in large plantations.

It ensures that all operations are synchronised in the plantation such that, plants would emit inflorescences at the same period and crop harvesting would be synchronised and made easy 352. In large plantations containing genetically uniform monoecious-allogamous trees yielding to a common prevailing weather condition, crop production may decline because flowering emergence, responding to environmental conditions shall also be uniform and synchronised. This implies that when climatic conditions are favouring the emergence of female inflorescences, all trees will be carrying female inflorescences and there shall be lack of male inflorescences to pollinate them.

This will lead to poor fruit set and inflorescence abortion, leading to low production. It would be preferable to mix a few genetically different tree varieties in each block during planting. The phenology of these few genetically different trees may not synchronise with the majority of trees, and therefore they may be producing male inflorescences while the others are producing female inflorescences. Pollination and fruit set can therefore be optimal in such a design.

4.1 Functional analysis of genes regulating inflorescence emergence

We used a co-expression network analysis in GeneMANIA 41 to find out the functional networks in which both down regulated and up regulated gene sets are involved. Table 3 shows the different functional networks for down regulated and up regulated genes.

Table 3: Functional networks involving down regulated and up regulated DEGs.

Down regulated DEGs	Up regulated DEGs
Cellular response to P-starvation	Response to high light intensity
Response to nutrient levels	Response to hydrogen peroxide/ROS
Cellular response to external stimulus	Response to heat
CHO derivative biosynthesis	Response to light intensity

Down regulated genes were mostly involved in response to nutrient levels and starvation while up regulated genes were mostly responding to light intensity, Reactive Oxygen Species (ROS) and heat stress.

The light receptors *PHYTOCHROME A (PHYA)* and *CRYPTOCHROME 2 (CRY2)* are stabilisers of the *CONSTANS (CO)* genes that regulate light dependent flowering time in Arabidopsis 46216.

Hence, it is understandable to observe that a majority of the up regulated DEGs are related to light response and management. These up regulated DEGs are in majority photosynthetic genes working to produce more carbohydrate while the down regulated genes working to efficiently use the limited amount of carbohydrate. Lee *et al.* 26 observed an abundance of photosynthesis related genes in previously defoliated plants of *Lolium perenne*. Bartos *et al.* [9 and Baptist *et al.* 7 reported an increase in carbohydrate concentrations in plant organs before winter than at the peak of the growing season.

The down regulation of carbohydrate metabolism genes in flower tissues during stress has also been reported 30. It is also known that carbohydrate related hormones such as *HEXOKINASE 1 (HXK1), PHOSPOGLUCOMUTASE 1 (PGM1), TETREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1) and INVERTASE (INV)* have been shown to regulate flowering time 39. Alterations in climatic factors such as rainfall, soil water availability; light, and temperature reduce carbohydrate supply to flowering meristem thereby regulating flower induction, emergence and maturity 4445263223.

The balance between carbohydrate production from source organs such as leaves and carbohydrate consumption in sink organs such as flowers and fruits is greatly affected by seasonal changes. Thus, the prevailing environmental factors during the wet season affect the balance of carbohydrate in such a way that it favours the emergence of male inflorescences.

4.2 Functional classification based on Gene Ontology

Gene set enrichment analysis was performed on DAVID 22 to verify that genes differentially expressed between the defoliated and non defoliated tissues were functionally relevant to flowering time. Gene set functional annotation clustering, evaluated based on the enrichment score (ES) and the p-value produced a total of 157 clusters. The highly enriched clusters were composed of genes involved in cellular response to stress (ES=3.2%), response to temperature stimulus (ES=2.32), response to far red light *FAR* (ES=1.7%), response to starvation (ES=1.47%) and response to carbohydrate stimulus (ES=1.34%). The ES for these gene groups signifies their functional implications during response to complete defoliation stress. In the much-studied Arabidopsis, individual pathways that include photoperiod 39 vernalisation 25, autonomous 33, ageing 20 and GA 34, regulate flowering time. Response to light, temperature and carbohydrate metabolism were among the functional gene clusters that were highly enriched in our study. These findings indicate that genes related to carbohydrate supply to flowers, temperature and light regulate flowering emergence in the perennial tree oil palm. Sample tissues from this study were collected from oil palm tree, which is a tropical tree crop that may be neutral to day length.The light related gene, Far-red impaired response (*FAR1*) that regulates flowering time in response to various biotic and abiotic signals was highly up regulated with a fold change of 17 in our experiment. *FAR1* has been shown to regulate flowering in photoperiodic neutral plants. *FAR1* Knock out mutants, flowered early regardless of day length suggesting that *FAR1* regulate flowering time irrespective of photoperiod. Water availability plays important role in the hydrolysis of carbohydrate. Mclaughlin and Boyer 30 demonstrated that water deficit affected glucose supply to plant ovaries. Moreover, in the presence of water, sugar is converted into hexoses by *invertase* and *sucrose synthase*. The seasonal fluctuation of carbohydrate concentration in plants has been shown to affect flowering time 23. Much of the carbohydrate in the oil palm is seasonally depleted due to the export of its energy through fresh fruit bunch harvests 15, which occurs during the dry season. Fresh fruit bunches are borne by female inflorescences. Bunches from a mature tree consume much energy and bunch number varies between 8 and 21 per tree with average weights of between 9-12 kg 36. Also, each fresh fruit bunch takes five months between fertilisation and harvest from the tree. The male inflorescences from a mature tree weighs just between 1-2 kg and they get into senescence one month after emergence. Thus the less-energy-demanding male inflorescences appear during the following wet season after the tree energy has been considerably depleted. The wet season is thus used to recover from the energy loss and also make produce new energy through photosynthesis in preparation for the heavy production dry season.

5. CONCLUSION

Inflorescence emergence in the oil palm, a perennial tree crop, is pre-programmed and inflorescences appear based on signals from seasonal and environmental cues. Voluntary inflorescence induction in oil palm through complete defoliation stress does not affect the seasonal trend of inflorescence emergence and the time of inflorescence emergence is independent of its induction date. Male inflorescence emergence was more significant in the dry season than the wet season. Genes responsible for carbohydrate metabolism, temperature and light intensity may be responsible for the regulation of inflorescence emergence time in oil palm. When these factors prevail, genetic pathways shall immediately be engaged, and inflorescences are emerged. Consequently there should be flowering earliness and lateness in the oil palm tree crop. Knowing the factors controlling inflorescence emergence, crop production estimation and breeding strategies can efficiently be designed by agronomists and breeders.

6. ACKNOWLEDGEMENTS

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