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Research Article

Influence of Heat and Water Stress around Meiosis on Pollen Quality in Two Pollen Parents of Coconut (*Cocos nucifera* L.) Used in Control Hybridization in Sri Lanka

KGAPK Amarasinghe[1]*, CS Ranasinghe[2], DC Abeysinghe[1] and AAFLK Perera[2]

[1]Department of Plantation Management, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka

[2]Coconut Research Institute, Lunuwilla, Sri Lanka

***Corresponding Author:** KGAPK Amarasinghe, Department of Plantation Management, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.

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Abstract

Climate resilient and high yielding Dwarf x Tall hybrids is the main strategy to meet increasing demand of coconut in a changing climate where sexual reproduction is susceptible to heat and drought stress (HTDS). Fruit set failures are mainly attributed to high sensitivity of pollen and ovules to stress, particularly around meiosis, in many crops. This study focused to assess the influence of heat (monthly mean Tmax > 33^0C) and water stress (monthly rainfall < 90 mm) around meiosis on the quality of pollen in San Ramon (SR) and Sri Lanka Tall (SLT) cultivars that used in controlled hybridization. We measured carbohydrates, germination (PG%) and tube length (PTL) in pollen developed under heat and/or drought stress and unstressed (control) around meiosis (final four months prior to flower opening).

San Ramon showed significantly higher PG% and PTL (43%, 517 µm respectively) compared to that of SLT (37%, 481 µm respectively) cultivar. Unstressed male flowers had significantly higher PG%, PTL and starch compared to flowers in stress at any stage around meiosis. The cumulative rainfall, number of dry days (rainfall < 3 mm/day), total number of heat stress days (Tmax < 33^0C) and average maximum temperature during the period around meiosis had merge impact on pollen starch (R^2 = 0.96), total soluble sugar (TSS) (R^2 = 0.71), pollen germination (PG%) (R^2 = 0.61) and pollen tube length (PTL) (R^2 = 0.89). The study concluded that heat and/or water stress around meiosis is very critical for pollen quality in two tested cultivars of coconut. This information will be of great importance to manipulate the pollination strategy to minimize stress-affected fruit set failures under controlled pollination.

Keywords: Coconut Hybrids; Flower Carbohydrates; Fruit Set; Heat and Water Stress; Pollen Germination

Abbreviations

HTDS: Heat and Drought Stress; PTL: Pollen Tube Length; PG%: Pollen Germination Percentage; SR: San Ramon; SLT: Sri Lanka Tall; TSS: Total Soluble Sugars

Introduction

With the increasing global demand for coconut products, all coconut growing countries encounter a considerable gap between coconut production and the requirement to meet the demand and, this gap is being widened due to the effects of climate change. Production of hybrid coconuts between dwarf (nana) and tall (typica) varieties are highly successful in terms of higher yields compared to pure tall forms [1]. These dwarf x tall hybrids are developed under controlled pollination of emasculated (male flowers removed) dwarf palms with pollen of tall varieties under field condition. The

optimum temperature for pollen germination of coconut is 28 - 29 ^{0}C [2] and well distributed rainfall greater than 1800 mm per annum. The critical temperature and rainfall for reproductive success in coconut is 33^{0}C and 90 mm/month (considering the water requirement of 3 mm/day), respectively, and the months with Tmax (monthly maximum temperature) exceeding 33^{0}C and rainfall lower than 90 mm cause heat and/or water stress on the developing reproductive organs [3,4]. The coconut palms grown in tropical countries like Sri Lanka are frequently exposed to these stress levels. In warm/drought seasons significant failures in fruit set of the hybrid seed nuts occur especially in flowers opening when the sensitive stages of the inflorescence development are exposed to these stress conditions are already witnessed in the coconut seed gardens. The frequency of these extreme events is in the increasing trend due to climate change [5].

Although the coconut inflorescence development from flower primodium to mature nut is a 27 - 28 month long process [6], short-term climatic variability of 2-3 months prior to inflorescence opening has a vital role in determining fruit set in coconut. Coconut palms produce approximately one inflorescence a month. A mature inflorescence consists of approximately 30 rachillae and each rachilla carries both male and female flowers, the former at the upper end and the latter at the base. The most important development stage, sex determination of the flowers, takes place in the 4th months prior to inflorescence opening (-4 month) [7]. Thereafter, the critical development stages of male flowers take place during the final three months; differentiation of anther (-3 month, 3 months prior to opening), meiosis of microspore mother cells (-2 month), pollen mitosis (-1 month) and pollen maturation (0 month, month of flower opening) [7].

The reductions in hybrid fruit setting can be due to reduced quality of pollen, female flowers or impaired pollination process and this paper mainly address the effect on pollen. During controlled pollination process, once the pollen is applied on the pistil of the receptive fertile flowers, pollen germinates on the pistil and the pollen tube growth is fueled by the existing carbohydrate reserves in the pollen grain [8]. Heat and water stress around meiosis stage of pollen can alter the carbohydrate content of pollen grains inhibiting male and female gametophyte development, pollen germination, pollen tube growth and fertilization [9-11]. There is considerable number of reports published on the effects of either heat or water stress around meiosis on reproductive development of crops under controlled conditions [9, 12-15]. However, the studies under field condition are limited and none so far in coconut.

Mature coconut pollen can be collected, processed under vaccum and stored under low temperature for nearly one year without affecting its quality [2]. Therefore, if detailed information on the influence of climatic variability around meiosis on the fertility of pollen is identified, a pollination strategy to minimize stress-affected failures in hybrid seed set can be developed. So far, such attempt has not been taken for coconut. This study, therefore, focused to assess the possible influence of heat and/or water stress around from meiosis to maturation of pollen (under field conditions) on the quality of pollen (pollen germination ability, measured as (PG%), pollen tube length (PTL), total soluble sugar (TSS) and starch) in two commonly used pollen parents; Sri Lanka Tall (SLT) and San Ramon (SR) for controlled hybridization programmes in Sri Lanka. Since the palms were managed with similar agricultural practices throughout the experimental period and the solar radiation intensity was always above the sufficiency level for coconut, we hypothesized that the pollen quality parameters of the two varieties may vary in response to heat (monthly mean Tmax > 33^{0}C) and water stress (monthly total rainfall < 90 mm) prevailed around the meiosis stage of male flowers (final four months prior to flower opening).

Materials and Methods

Site and plant materials

Adult coconut (*Cocos nucifera* L.) palms of two tall forms (Sri Lanka Tall (variety *typica,* SLT) and San Ramon Tall (variety *typica*, SR)) which are currently being used as pollen parents for production of CRIC65 (Sri Lanka Dwarf Green x SLT) and Kapruwana (Sri Lanka Dwarf Green x SR) in seed gardens of the Coconut Research Institute of Sri Lanka (CRISL) (latitude 7° 07' N, longitude 79° 87' E) were used. The experimental site is located in the low country intermediate zone (IL_{1a}), according to the classification of Agro-Ecological Regions of Sri Lanka [16]. Generally, these areas receive the highest rainfall during October to December and are prone to moderate to severe droughts during February - March to September and August periods [17]. The plantations were maintained with agricultural practices recommended by CRISL. Twelve representative tall palms (six each from SLT and SR (pollen parents)) were selected for pollen sampling and data was collected in eight selected stress levels.

Collection of flowers developed under different stress levels

Months of sample collection were selected based on the historical climatic data on rainfall and maximum temperature prevailed in the experimental site over last 30 years and monthly fruit set and yield data in the experimental area over last five years. Accordingly, sampling of male flowers was done at eight occasions to represent flower development under different stress levels at the stages around pollen meiosis to pollen maturation – (final four months prior to opening). Six occasions with heat and/or water stress at stages around meiosis (flowers opened in March, June and September) and two occasions without heat or water stress (controls) around meiosis (flowers opened in December) were selected in three consecutive years (Table 1).

Collection and processing of pollen

Spikelets with ready-to-open male flowers were sampled from SLT and SR palms between 9.00 - 10.00 am [2] and processed to obtain pollen using fluidized bed dryer (FBD) and immediately stored at -4°C until analysis. Pollen germination (PG%), pollen tube length (PTL), total soluble sugar (TSS) and starch concentration of pollen grains were measured (details are given below).

In-vitro pollen germination (PG) and pollen tube growth (PTL)

Pollen was dusted into micro centrifuge tubes containing 500 µL of germination medium [2]. One set of tubes containing pollen and germination medium was incubated at 28^0C for 3 hrs (for measurement of PTL) and the other set of tubes were incubated at 28^0C for 24 hrs (for %PG). The length of pollen tubes was measured with an ocular micrometer fitted to the eye-piece of the microscope (×40 magnification). Numbers of germinated and non-germinated pollen in a microscopic field were counted to calculate the PG %. For estimating the PG% and PTL of each month, nine microscopic fields/palm (54 fields each from SLT and SR) and 18 pollen tubes/palm (108 tube lengths each from SLT and SR), respectively, were used.

TSS and starch in mature pollen

The TSS of pollen (0.1g) were extracted with 80% ethanol and concentrated supernatants were analyzed for total soluble sugars using Phenol Sulfuric Method [4]. The residue was digested using 1% α-amylase and the starch content (sugar equivalents) was measured by the same photometric method at 490 nm wavelength (UV/VIS Spectrophotometer).

Climate and other supportive soil and plant data

Daily rainfall and temperature were collected from the Agri-Meteorological Station at the experimental site. Of the temperature components, the maximum temperature (Tmax) was found to be the most influential on coconut fruit setting [4] and therefore, monthly mean of Tmax values and monthly total of rainfall were considered as the mean parameters causing climatic variability among months. Soil moisture percentage adjacent to experimental palms (six points/variety, SM%, at 25 cm depth by gravimetric method [18]) and leaf stomatal resistance of palms (six palms/variety by LI-COR photosynthesis meter), the most appropriate indicators to express the stress perception by palms, were measured during the eight sampling months to ascertain the mean soil and plant stress levels at sampling.

Data analysis

The main analysis, statistical differences of measured parameters among the eight months (flowers exposed to six different stress levels and two control conditions) was conducted by Analysis of Variance (ANOVA) following the General Linear Model (GLM) procedure using the SAS statistical package version 9.1. Means were separated using Duncan's New Multiple Range Test. The differences between two varieties within each month were compared by a t test. Regression analysis was performed to analyse the relationships between tested parameters with climatic variables.

Results

Climatic conditions during flower development

The monthly maximum temperature (mean Tmax) and total rainfall during the experimental period is shown in figure 1a and 1b. The Tmax was higher than the critical limit (33°C) during March and April and the rainfall during September to December was always higher than the critical limit (90 mm /month) in all three years. Accordingly, the eight stress levels at the stages around meiosis and maturation of pollen are depicted in table 1.

The variation in soil moisture (SM%) of the plantation at eight sampling occasions clearly showed the intensity of stress prevailed due to rainfall and temperature during respective months. Leaf stomatal resistance (*rs*), the best indicator of palm water stress, also revealed the different intensities of stress levels perceived by the palms at eight sampling months (Table 1, Figure 1c). In December (control flowers), the palms had higher SM% and lower *rs* compared to that of all other sampling months. The SM% negatively correlated with the stomatal resistance of palms ($R^2 = 0.51$, $p \leq 0.05$) (Figure 1d).

Variation of pollen germination ability (PG%) and pollen tube growth (PTL)

There was a significant ($p < 0.05$) difference in PG (%) and PTL between flowers stressed and non-stressed (control) pollen during the sensitive stages (around the meiosis to maturation stage)

and both varieties showed the similar pattern of variation (Figure 2a and 2b). For instance, pollen developed without stress during all four months around meiosis (control) (pollen of December 2013 and 2014) had significantly higher PG (50 - 60%) and PTL (638 µm) than those developed under stress at any stage around meiosis (pollen of March, June and September of both years). Of the stressed pollen, the PG (%) and PTL varied according to the stage of exposure to stress, though the pattern of response was not the same. PG% showed a very high sensitivity to continuous water stress at pre-meiosis [19], meiosis and post-meiosis stages (pollen produced in September 2013, Table 1), and had the lowest PG (18%). Further, PG% and PTL showed positive correlation with the total rainfall received to the experimental area (ISG) during pollen meiosis to pollen maturation period (Figure 2c and 2d).

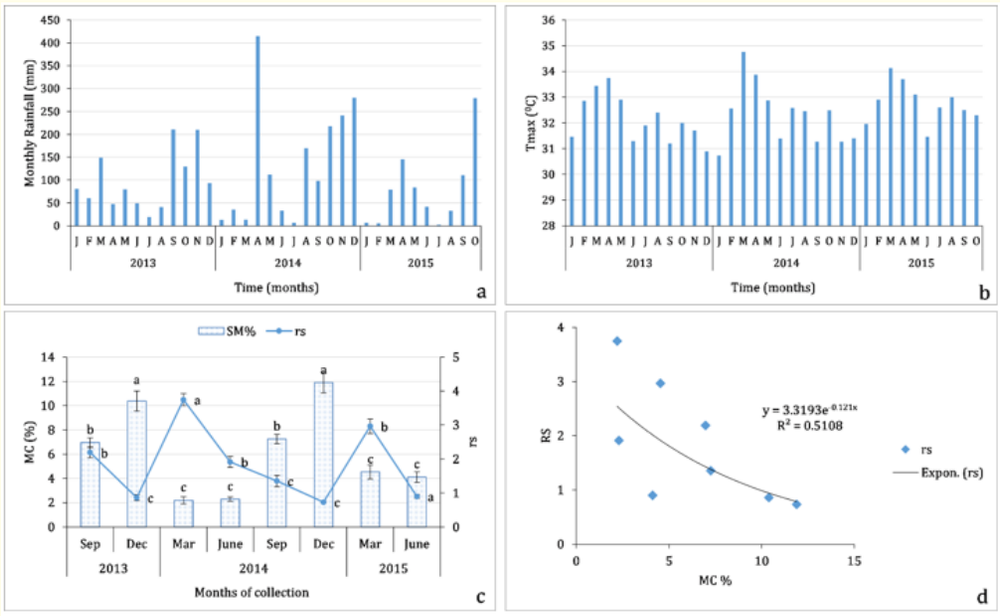

Figure 1: Variation in (a) monthly total rainfall (mm), (b) maximum temperature (Tmax, °C), (c) soil moisture percentage (MC%) along with stomatal resistance (RS) in the experimental area (ISG)during the experimental period (2013 - 2015) and (d) correlation between MC% and RS. Means with the same letter in one variable (MC%/RS) are not significantly different at $P < 0.05$, bars indicate ± SE in figure 1c. SM: soil moisture %, RS: stomartal resistance in figure 1c and 1d.

Month of sampling of newly opened flower	-3 stage		-2stage		-1stage		0 stage	
	HS	WS	HS	WS	HS	WS	HS	WS
Sep 2013								
Dec 2013								
Mar 2014								
June 2014								
Sep 2014								
Dec 2014								
Mar 2015								
June 2015								

Table 1: Description of the exposure of pollen to heat and water stress during in eight sampling events (months) based on rainfall and maximum temperature (Tmax) during critical period of their development (from meiosis to maturation of pollen).

0 stage: month of flower opening (pollen maturation), -1 stage: first month prior to flower opening (pollen mitosis), -2 stage: second month prior to flower opening (pollen meiosis), -3 stage: third month prior to flower opening (anther dehiscence)). WS: Water stressed (rainfall < 90 mm/month) and HS: Heat stressed (Tmax ≥ 33°C) stages are highlighted (horizontal bars).

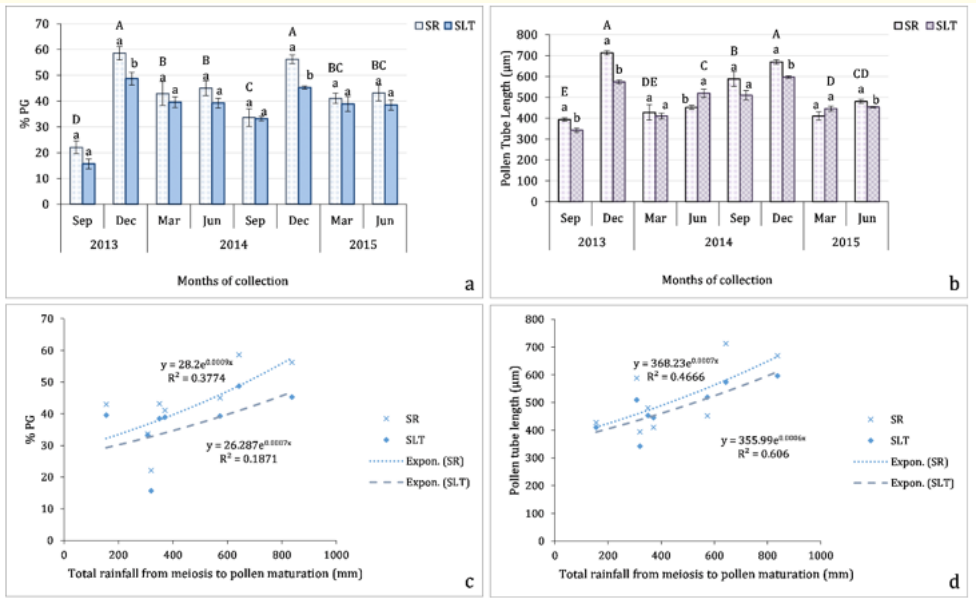

Figure 2: Pollen germination ability (PG %) (a), pollen tube length (PTL) (b), correlation between pollen germination ability (PG%) and the total rainfall from pollen meiosis to pollen maturation (c) and correlation between pollen tube length (PTL) and the total rainfall from pollen meiosis to pollen maturation (d) of mature pollen grains of SR and SLT varieties collected in different months. Capital letters indicate significance among the months of sampling and lowercase letters between varieties within a month. Means with the same letter are not significantly different at $P < 0.05$, bars indicate ± SE.

Variation of starch and total soluble sugars (TSS) in pollen

Starch of pollen showed a significant variation not only in response to water and/or heat stress around meiosis but also to their intensity (Figure 3). For instance, the pollen produced under no stress (in December) had significantly higher starch contents compared to all other stressed flowers around meiosis (except the pollen starch in September 2013), and there was a significant difference between two controls; pollen starch in December 2013 and 2014; the former was higher than the latter. This may be mainly attributed to the intensity of rainfall at pre-meiosis stage of pollen produced in December (higher in 2013 than 2014) (Figure 1). Similarly, the pollen produced under stress in March, June and September in 2014 had lower starch content than the pollen produced in the same months in other two years. This may be mainly attributed to the higher heat stress (35^{0}C) at spore maturation stage (0 month, Table 1) of March flowers, the same heat stress at pre-meiosis stage of June flowers (-3 month) and, higher water stress at meiosis and pre-meiosis stage of September flowers in 2014, compared to flowers opened in the same months in other two years (Figure 1).

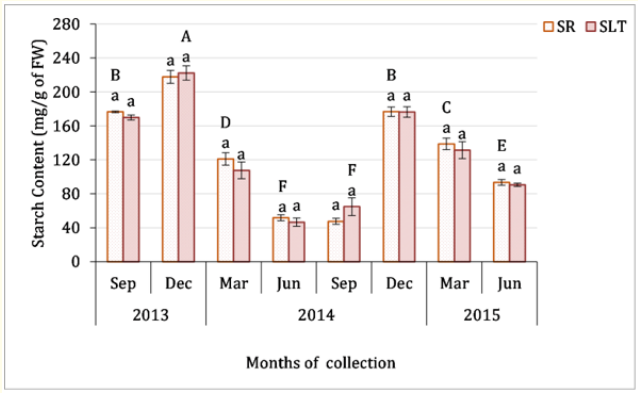

Figure 3: Starch concentration of mature pollen grains of SR and SLT varieties collected in different months. Capital letters indicate significance among the months of sampling and lowercase letters between varieties within month. Means with the same letter are not significantly different at $P < 0.05$, bars indicate ± SE.

Pollen exposed to water stress at the stage of meiosis (-2 month, Table 1) (flowers of March and September), had higher TSS compared to flowers that were not exposed to water stress at meiosis (flowers of June and December). Further, when pollen was not water stressed at meiosis, the TSS of pollen was not significantly different to each other (flowers of June and December), irrespective of heat or water stress at other sensitive stages, or even heat stress at meiosis (June pollen) (Figure 4).

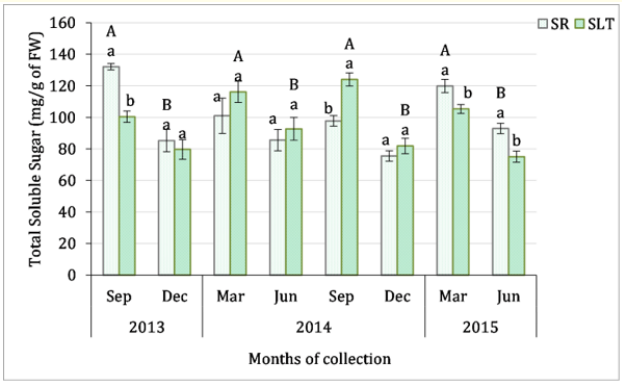

Figure 4: Total soluble sugar (TSS) concentration of mature pollen grains of SR and SLT varieties collected in different months. Capital letters indicate significance among the months of sampling and lowercase letters between varieties within month. Means with the same letter are not significantly different at $P < 0.05$, bars indicate ± SE.

Multiple linear regression analysis of climate data with pollen quality

The multiple linear regression showed that, cumulative rainfall (TRF), number of dry days (TDD) (rainfall < 3 mm/day), total number of heat stressed days (THD) (Tmax ≥ 33^0C) and average maximum temperature (Tmax avg) during the sensitive period from meiosis to pollen maturity (final four months) had compound impact on pollen starch ($R^2 = 0.96$), total soluble sugar (TSS) ($R^2 = 0.71$), pollen germination (PG%) ($R^2 = 0.61$) and pollen tube length (PTL) ($R^2 = 0.89$).

TSS = - 1366 - 0.0836 TRF - 0.550 TDD - 1.49 THD + 49.9 Tmax avg

Starch = 4058 + 0.0470 TRF + 0.749 TDD + 1.15 THD - 126 Tmax avg

PG% = 936 + 0.0639 TRF + 0.840 TDD + 0.908 THD - 32.3 Tmax avg

PTL = 10311 + 0.839 TRF + 11.9 TDD + 8.41 THD - 362 Tmax avg

Discussion

The present study revealed that when coconut pollen developed without heat or water stress at the stages from pre-meiosis to pollen maturation, (four month period prior to flower opening, Table 1), those pollen had the highest germination ability (%), pollen tube length growth and pollen starch contents. When there was a heat and/or water stress at any stage during the above period, (Table 1) it reduced the pollen germination (%), pollen tube growth and pollen starch. In the stressed flowers, water stress at the stage of meiosis (-2 month) increased the TSS and when pollen developed without water stress during meiosis stage (-2) the total soluble sugar (TSS) content was low.

There can be several reasons for the reduction in pollen quality under heat and/or water stress. Since various phases of pollen development (differentiation of anthers, meiosis of mega-spore mother cells, pollen mitosis and pollen maturaion) are sensitive to water stress [12], any factor that affects the pollen formation and function may have caused loss of pollen germination ability. For example, in the present study the most deleterious effect on PG (%) was shown when there was a continuous water stress during pre-meiosis (-3 month, Table 1), meiosis (-2 month) and post-meiosis (-1 month) stages. That water stress may have altered the structural development of anthers such as tapetum and middle layer, subsequently limiting the nutrition supply to developing pollen grains and reducing the fertility of pollen at their maturity [20].

Another possible reason for reduced pollen quality is the changes in carbohydrate metabolism. Sucrose, which is the principal sugar (TSS) transported to developing pollen, is generally converted to hexoses by invertase and / or sucrose synthase and these resulting hexoses are used for starch synthesis which will be later used as energy source for pollen germination [21]. In the present study, it was clearly shown that, water stress at the stage of meiosis of pollen mother cells, appreciably increased the TSS in pollen irrespective of the stress levels at anther differentiation or mitotic stages. Therefore, it can be suggested that water stress mainly at the stage of meiosis of mega–or micro spore mother cells (-2 month) may have reduced the activity of acid invertase and thereby accumulated sucrose (the main component of TSS) while restricting the starch biosynthesis and other critical processes involved in pollination. The study also revealed that the reduction of pollen-starch with increasing mean Tmax during the stages around meiosis, this may also support the reduction of acid invertase and/or starch syn-

thase enzyme activity under heat stress. Similar results have been observed in previous studies. In rice anthers, water stress during meiosis inhibited starch accumulation, enhanced the accumulation of soluble sugars and induced male sterility [21,22], in barley grains from heat- treated plants accumulated less starch due to reduced conversion of sucrose to starch [23] and cotton flowers exposed to moderately high temperatures showed reduced carbohydrate reserves in the female flowers (pistils) [11]. The study also highlighted that the response of coconut reproductive organs to heat stress are not necessarily the same as their response to water stress. This needs to be further evaluated.

It is also possible to argue that since the soil moisture condition during stressed months was comparatively low and leaf stomatal resistance (for water vapour) was high, there could be a possible reduction in plant assimilate production during the stressed months, and it may have also contributed to low starch accumulation in stressed pollen and female flowers. This reason could not be eliminated; however, the variation in rate of leaf photosynthesis of these palms during the sampling months did not follow the same pattern as the variation in pollen carbohydrates (data not shown). In addition there are reserved carbohydrates in coconut stem and fronds, which may be used by the reproductive organs in the periods of short supply [24]. Therefore, these overall results suggest that carbohydrate starvation was probably not the main factor responsible for the meiosis stage stress-induced low flower quality in coconut, and it could be the carbohydrate metabolism as observed in many other crops [9]. In addition, the limitations to carbohydrate translocation from storage organs to reproductive sinks due to callus formation under stress could not be eliminated [9]

This information can be used to predict the quality of pollen produced in a particular month. In the production of hybrid seed coconuts by controlled pollination in drought prone areas, the pollen produced without any stress at the final four months prior to flower opening, could be used for pollinating the female flowers produced during stressed months, instead of using the stressed pollen produced in the same month (which is the current practice). This would definitely minimize the heat and drought affected fruit set failures in hybrid coconut production and this concept is now under evaluation.

Conclusion

Pollen quality is an important parameter to be considered in successful fruit set and it can be significantly affected by climatic variability. Water and/or heat stress at the stages (from anther differentiation to pollen maturation) of pollen altered total soluble sugars (TSS), starch, germination (%) and tube growth of pollen. Pollen developed without heat or water stress around these stages had the best quality pollen.

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