

Approach to Screen Coconut Varieties for High Temperature Tolerance by *in-vitro* Pollen Germination

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ABSTRACT

Successful fruit set in coconut depends on several reproductive processes including pollen quality, pollen germination percentage (%PG) and pollen tube growth processes. Three preliminary experiments were conducted at the Coconut Research Institute, Lunuwila, Sri Lanka to quantify the response of *in vitro* pollen germination in coconut to time of incubation, the response of *in vitro* pollen germination and pollen tube growth to temperature and to determine the cardinal temperatures (T_{min} , T_{opt} , T_{max}) of coconut varieties for *in vitro* pollen germination. Pollen was collected from palms of six cultivars / varieties expressing variable tolerance to abiotic stresses. Pollen germination and pollen tube length were recorded after incubation in artificial growth media at the respective treatments. For pollen collected during October / November periods, cultivars differed for *in-vitro* pollen germination percentage with a mean values of 23%. The mean cardinal temperatures (T_{min} , T_{opt} , T_{max}) averaged over cultivars were 20.0 °C, 28.0 °C, and 38.8 °C, respectively, for pollen germination. The information generated in this study will be tested in different seasons of the year for consistency and used to develop a heat tolerance index for coconut. This index will be used to identify coconut varieties suitable for high-temperature prone areas. In addition, the identified cardinal temperatures and response functions could be incorporated into process-based coconut models to increase prediction accuracy under current extreme and projected future climates.

Key words: *Cocos nucifera*, pollen germination, heat stress, cardinal temperature

INTRODUCTION

Sexual reproduction in plants is more sensitive to high temperature than vegetative processes, and therefore, plant reproductive organs will be more vulnerable to changes in short episodes of high temperature prior to and

during early flower stage. Fruit set in cowpea (Ahmed and Hall, 1993), cotton (Reddy et al., 1997), ground nut (Prasad et al., 2003) and tomato (Sato et al., 2002) is sensitive to high temperatures. Furthermore, plant reproductive

organs will be more vulnerable to changes projected in climate such as increase in Earth's surface temperature to anywhere between 1.5 and 11 °C by 2100 due to increases projected in greenhouse gases (IPCC, 2007). In addition, short episodes of extreme events including high temperatures projected to occur more frequently in the future climate will impact fruit set and yield. Therefore, it would be advantageous for plants to exhibit greater reproductive survivability at extreme temperatures normally encountered during plant reproduction and for processes leading to yield such as pollen grain development, pollen germination, pollen tube growth, fertilization and embryo development. Two stages of pollen development, microspore mother cell meiosis (Sato et al., 2006) and mature microspores at anthesis have been reported to be highly sensitive to high temperature (Erickson and Markhart, 2002). The major cause of low pollen fertility under high temperature was reduced pollen germination (Sato and Peet, 2005).

The coconut inflorescence bears a large number of spikelets with male and female flowers. The female flowers are situated towards the base of each inflorescence and above them are a large number of closely arranged male flowers. Inflorescences open successively at intervals varying from 22 to 30 days, depending on the environmental condition and age of the palm. From the second to the nineteenth days after the opening of the inflorescence, the male flowers open, liberate pollen and fall off. Open male flowers do not remain on an inflorescence for more than one day, generally open in the early hours of the day and are shed the same evening. The male flowers near the apex of each

spikelet open earlier than those in the middle. Pollen grains remain viable only for two days under atmospheric conditions (Liyanage 1954). In the CRI coconut breeding programme, Tall and San Ramon varieties are the two main pollen parents. Of the recommended varieties, Tall variety is the pollen parent of Ambakelle Special, Ambakelle Tall (CRIC60), Dwarf Green x Tall (CRIC65), Dwarf Yellow x Tall (CRIC65) whilst San Ramon is the pollen parent of Tall x San Ramon (CRISL98), Dwarf Green x San Ramon (Kapruwana) and varieties such as Dwarf Brown x San Ramon that is to be recommended within next one to two years. Reduced fruit set in coconut under high temperatures are often experienced in plantations in the dry-intermediate and dry zones of Sri Lanka. This could be mainly attributed to the fact that male flowers of coconut palms exposed to full sunlight experiencing temperatures as high as 35 °C – 39 °C which is well above the optimum temperature for pollen germination (25 °C -30 °C) in many field-grown crops (Burke et al., 2004). Therefore, it is imperative to develop tools for screening coconut for high temperature tolerance.

Pollen, once released from the anthers, acts as an independent functional unit. Several recent studies have used the *in vitro* pollen germination and pollen tube length under different temperatures to screen genotypes, cultivars or species of groundnut (Kakani et al., 2002), cotton (Kakani et al., 2005) and Capsicum (Reddy and Kakani, 2007) for high temperature tolerance. However, to date, there have been no studies conducted to specifically document cultivar variability in coconut for *in vitro* pollen germination and pollen tube growth

and tolerance to high temperatures. This type of pollen characteristics will provide useful insight into the reproductive tolerance of coconut to high temperatures. Therefore, the objectives of this study were to (1) quantify the response of *in vitro* pollen germination to time of incubation, (2) quantify the response of *in vitro* pollen germination and pollen tube growth of coconut varieties to temperature, and (3) determine cardinal temperatures (T_{min} , T_{opt} , T_{max}) of coconut varieties for *in vitro* pollen germination.

MATERIALS AND METHODS

Palm Selection: Three experiments were conducted with several coconut (*Cocos nucifera* L.) varieties differed in origin, stature, breeding behavior and tolerance to abiotic stresses (Table 1) to accomplish the above three objectives.

The palms were selected from experimental blocks of Genetics and Plant Breeding Division in Bandirippuwa Estate, Lunuwila (TT, SR, DR), Genetic Resource Centre, Ambakelle (DG) and Pottukulam Research Station (DB,

DY) of Coconut Research institute, Sri Lanka (latitude 7° 20' N, longitude 79° 53' E). The soils, management and cultural practices in all experimental plots were similar. Unless otherwise stated, the pollen was collected in October, November and December months in 2008 (for expt 1 & 2) and 2009 (for expt 3) (rainy season).

Experiment 1: The experiment was conducted to quantify the *in-vitro* pollen germination with respect to the time of incubation. Six palms of TT (commercially-grown variety) and San Ramon (exotic variety), the main pollen parents of the CRI breeding programme were selected randomly for pollen collection.

Experiment 2: This preliminary experiment was conducted to quantify pollen germination and pollen tube growth under different temperatures and to identify the temperature range that has to be tested for estimating the cardinal temperatures. Six palms of TT variety were selected randomly for pollen collection.

Table 1: Origin, stature, breeding behaviour and tolerance to abiotic stress of six coconut cultivars used in the study

Cultivar	Origin	Stature	Breeding behavior	Tolerance to stress
Tall x Tall (TT)	Sri Lanka	Tall (<i>typica</i>)	Cross pollinating	tolerant
Dwarf Brown (DB)	Sri Lanka	Dwarf (<i>nana</i>)	Self-pollinating	intermediate
Dwarf Green (DG)	Sri Lanka	Dwarf (<i>nana</i>)	Self-pollinating	sensitive
Dwarf Yellow (DY)	Sri Lanka	Dwarf (<i>nana</i>)	Self-pollinating	sensitive
Dwarf Red (DR)	Sri Lanka	Dwarf (<i>nana</i>)	Self-pollinating	sensitive
San Ramon (SR)	Philippines	Tall (<i>typica</i>)	Cross pollinating	tolerant

Experiment 3: The experiment was conducted with six locally available cultivars to quantify cardinal temperatures (T_{opt} , T_{min} , and T_{max}) for *in-vitro* pollen germination. Six palms of TT, SR, DB, DG, DY and DR varieties were randomly selected for pollen collection.

Pollen collection: There is a maturity gradient of male flowers from top to bottom of the

use for analysis. To minimize the variation of maturity and germination gradient of pollen along the spikelets (Table 2), male flowers from the middle of the spikelets were sampled for pollen collection. In addition, palm to palm variation also can be a significant source of variation in pollen germination measurements. Therefore, male flowers from all six palms per variety were pooled to one sample before using it for pollen

Table 2: Percentage germination of pollen collected from different parts of the spikelet of two varieties (pollen collected in April 2008).

Variety	Position of the spikelet	% pollen germination
TT	Top	68.17
	Middle	60.62
	Bottom	32.81
San Ramon	Top	77.70
	Middle	64.26
	Bottom	32.81

inflorescence and the spikelets (Liyanage, 1954). Therefore, as a primary study, few germination tests were carried out on few spikelets, using pollen from top, middle and bottom of the same spikelet of two varieties (TT and SR). Similar to the maturity gradient, there was a gradient in percentage pollen germination from top to bottom of the spikelets (Table 2).

Therefore, spikelets with unopened male flowers were randomly sampled from the middle of the inflorescence within three to eight days after opening of the spathe, between 9.00 – 10.00 am (Liyanage, 1950). Collected spikelets were immediately put in to labeled, clear polythene bags, brought to the Plant Physiology laboratory and kept under refrigerated conditions until

germination tests, to minimize the variation with palms and position of inflorescence or spikelets. Pollen was collected by slicing anthers using a needle.

Germination medium and in-vitro pollen germination: Pollen was allowed to germinate in microfuge tubes containing the growth medium and pollen from three male flowers were always introduced into one tube. Pollen germination was analyzed as described by Pressman et al., (1998). Pollen was dusted into a tube containing 0.5 ml of germination solution consist of 100 g L-1 sucrose, 2 mM boric acid, 2 mM calcium nitrate, 2 mM magnesium sulfate and 1 mM potassium nitrate.

Experiment 1: In-vitro pollen germination with time

Five microfuge tubes of pollen per time period per variety were used for the germination test. The tubes containing pollen and germination media were placed at room temperature (around 27.5 °C) for 24 hrs. Number of germinated and non germinated pollen was recorded at one hr intervals from the time when pollen was placed on the germination media to eight hrs, then after 16 hrs and 24 hrs. A drop of germination solution was added on to a microscope slide, mounted a cover slip and counts were made under a low power light microscope (x10) (Olympus Optical Co., Tokyo, Japan). A pollen grain was considered germinated if it produced a tube longer than the diameter of the grain (Kakani et al., 2002). For each time period, 15 microscopic fields prepared from five randomly selected tubes of pollen (3 slides were prepared from one tube) were used for the analysis. The pollen germination (%PG) was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains per field of view and expressed as percentage.

Experiment 2: In-vitro pollen germination and pollen tube growth with temperature

Microfuge tubes containing pollen and germination media were incubated at pre-determined temperatures from 20 °C to 37 °C for 24 hrs (five tubes per temperature regime). Incubators were maintained at treatment temperatures and the temperature of the growth medium was not measured and assumed equal to set temperature. After 24 hrs of incubation, 15 microscopic fields prepared from five microfuge tubes of pollen (3 slides were prepared from

one tube) were used to analyse percentage pollen germination (%PG) as explained under Experiment 1. The *in-vitro* elongation of pollen tubes was measured on germinated pollen grains after 24 hrs. An ocular micrometer fitted to the eye-piece of the microscope was used to measure pollen tube length under a high power (x40) microscope. Two pollen tube lengths per each microscopic field (30 pollen tubes per temperature regime) were randomly selected for taking measurements. Mean pollen tube length was calculated as the average length of 30 pollen tubes.

Experiment 3: Cardinal temperatures for in vitro-pollen germination

Five microfuge tubes containing pollen and germination media per temperature regime per variety were used to measure % PG. The tubes were incubated at pre-determined temperatures from 20 °C to 40 °C at 2 °C intervals for 24 hrs. % PG was determined according to Experiments 1 and 2. The maximum pollen germination recorded after 24 hrs of incubation, at each temperature, was analysed using linear and non-linear regression techniques commonly used to quantify pollen parameter response to temperature (Kakani et al., 2002; 2005; Reddy and Kakani, 2007). Quadratic, cubic or higher order polynomial and bilinear equations were applied to data to determine the best-fit model. Curve estimation and non-linear regression procedures in SPSS were used to estimate parameters of the fitted models. The bilinear equation (Equation 1) provided the greatest r^2 value and smallest root mean squared deviation for pollen germination and was used to estimate cardinal temperatures (T_{min} , T_{opt} and T_{max}) for pollen germination of all the varieties.

Levenberg-Marquardt algorithm was used to estimate optimum parameters by keeping sum of the squares of the deviations minimum.

$$\%PG = a + [b_1(t - T_{opt})] + [b_2(ABS(t - T_{opt}))] \quad \text{Equation (1)}$$

a , b_1 and b_2 : equation constants, t : the various temperatures at which pollen germination was carried out and T_{opt} : the optimum temperature for pollen germination.

Values of T_{max} (maximum temperature at which the pollen germination is zero) and T_{min} (minimum temperature at which the pollen germination is zero) were estimated using equations 2 and 3 from the constants in equation 1.

$$T_{min} = \frac{[a + T_{opt}(b_2 - b_1)]}{(b_1 - b_2)} \quad \text{Equation (2)}$$

$$T_{max} = \frac{[a - T_{opt}(b_2 + b_1)]}{(b_1 + b_2)} \quad \text{Equation (3)}$$

RESULTS

In vitro Pollen germination with time of incubation:

Time course of *in vitro* pollen germination following 24 hrs at room temperature revealed that pollen germination was apparent within the first 30 minutes of incubation on the media (data not shown). In both cultivars, pollen germination percentage (%PG) increased sharply during the first eight hrs and reached their maximum germination within 16 hr of incubation and the values were almost constant thereafter. There wasn't a significant difference

in % PG between varieties during the first eight hrs of incubation, however, after 16 hrs and 24 hrs the % PG was generally higher in TT variety compared to that of SR. The highest % PG in TT and SR was 55% and 45%, respectively after 24 hrs of incubation (Fig. 1).

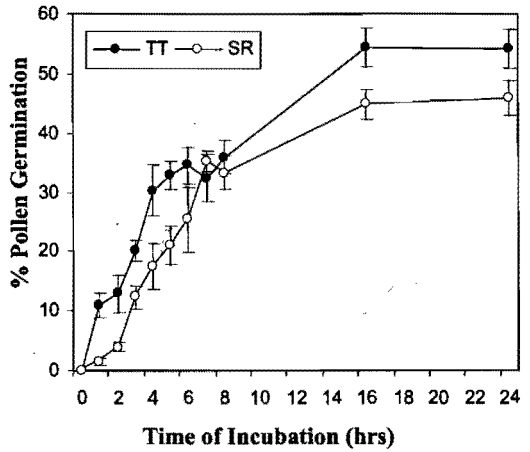


Figure 1: Mean (and standard error) percentage pollen germination of TT and SR varieties with time.

Response of pollen germination and pollen tube growth to temperature:

The preliminary experiment on pollen germination and pollen tube growth at different temperatures revealed that the maximum pollen germination and pollen tube length after 24 hrs of incubation were attained at temperatures between 27 °C and 28 °C. The percentage pollen germination was low at 20 °C and increased with increasing temperature up to 28 °C and thereafter, declined from a mean value of 38% at 28 °C to 9% at 37 °C. The pollen tube growth showed a similar trend with declining from a mean value of 663 μm at 27 °C to 217 μm at 37 °C (Fig. 2).

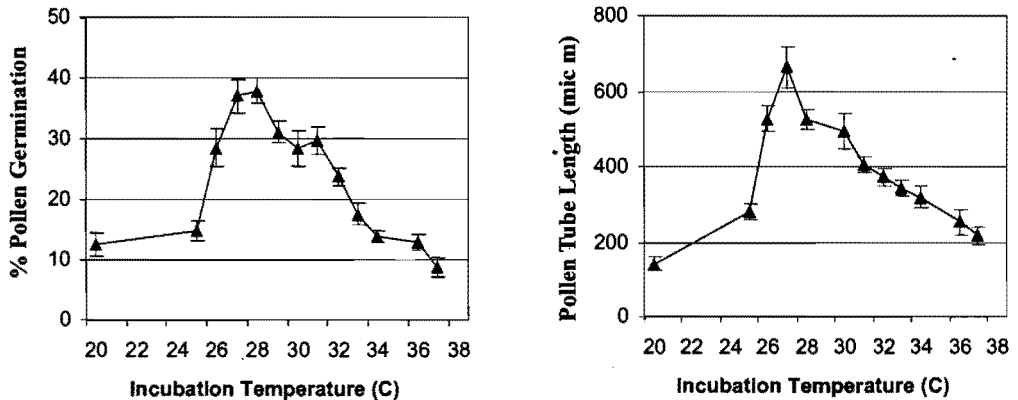


Figure 2: Response of % pollen germination (a) and pollen tube growth (b) to temperature

Cardinal temperatures for pollen germination

The bilinear equation (equation 1) described the response of pollen germination well, with r^2 ranging from 0.67 to 0.93. The maximum percentage germination recorded at optimum temperature ranged from 12% (DG) to 32% (SR), with a mean value of 23% (Table 3). The

cardinal temperatures differed greatly among the varieties. Values of T_{min} ranged from 18.3 °C (TT) to 21.2 °C (DR), T_{opt} from 26.0 °C (DR) to 30.0 °C (SR) and T_{max} from 37.5 °C (TT) to 40.4 °C (DG). The variety with highest % PG (SR) had the highest T_{opt} and it could maintain relatively higher pollen germination compared to other varieties even at 32 °C (Table 4, Fig. 3).

Table 3: Maximum pollen germination percentage and bilinear equation constants for pollen germination of six coconut cultivars in response to temperature

Cultivar	Maximum pollen germination (%)	Equation constants			
		a	b ₁	b ₂	r ²
Tall x Tall (TT)	29.18 ± 6.11	19.29	0.007	-2.008	0.71
Dwarf Brown (DB)	16.08 ± 2.95	8.81	0.207	-1.036	0.68
Dwarf Green (DG)	11.88 ± 1.89	11.63	0.279	-1.168	0.88
Dwarf Yellow (DY)	23.29 ± 2.18	26.39	0.163	-2.874	0.93
Dwarf Red (DR)	23.95 ± 5.49	15.55	1.031	-2.177	0.67
San Ramon (SR)	31.60 ± 4.10	21.22	-0.229	-2.384	0.74
mean	22.66				

Table 4: Cardinal temperatures for pollen germination of six coconut cultivars in response to temperature

Cultivar	Cardinal Temperature (C°)		
	T _{min}	T _{opt}	T _{max}
Tall x Tall (TT)	18.3	27.9	37.5
Dwarf Brown (DB)	20.9	28.0	38.6
Dwarf Green (DG)	19.3	27.3	40.4
Dwarf Yellow (DY)	20.3	29.0	38.7
Dwarf Red (DR)	21.2	26.0	39.6
San Ramon (SR)	20.2	30.0	38.1
mean	20.0	28.0	38.8

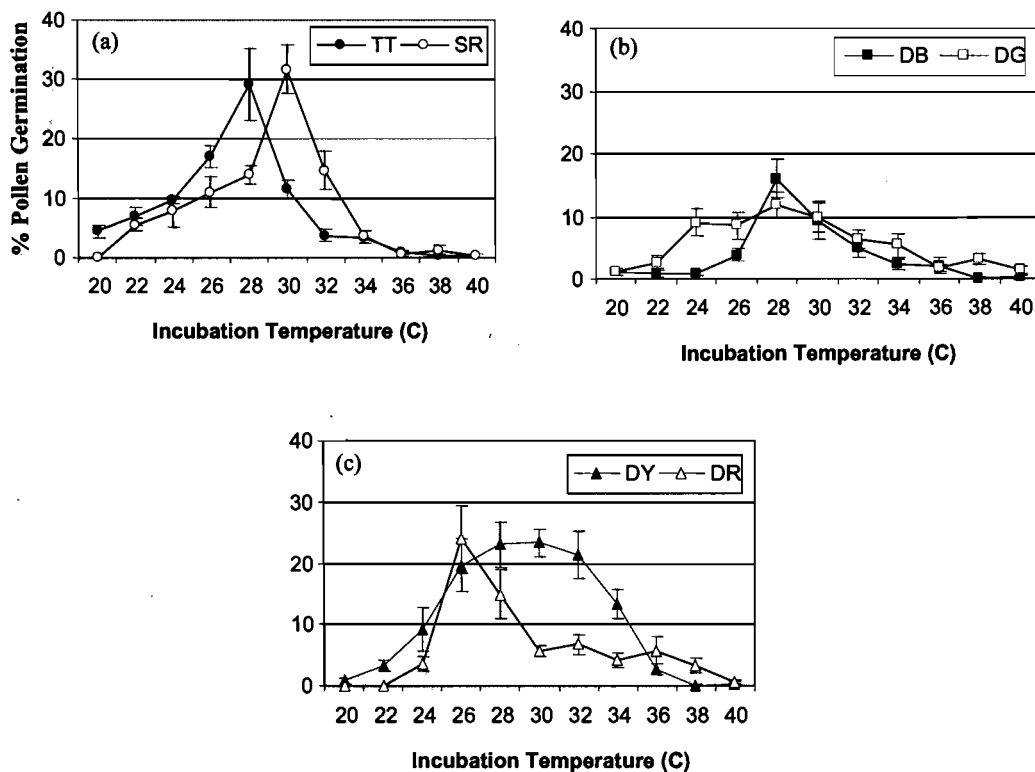


Figure 3: Response of pollen germination of coconut varieties [(a) TT and SR (b) DB and DG and (c) DY and DR] to temperature. Values are means and $\pm$ standard errors

DISCUSSION

Temperature is among the most important environmental factors affecting plant reproductive processes such as pollen germination, pollen tube growth and fruit set. In the present study, *in vitro* pollen germination and pollen tube growth severely reduced under both high and low temperature conditions and all six cultivars had defined temperature optima, above and below point of which pollen germination was reduced. The modified bi-linear model best described the response of pollen germination to temperature. Bilinear or modified bilinear regression models have been commonly used to study the response of *in vitro* pollen germination to temperature for screening crops for high temperature tolerance (Kakani et al., 2002; 2005; Reddy and Kakani 2007). Pollen germination percentages under artificial media observed for coconut (40%, Armendariz et al., 2006), groundnut (56%, Kakani et al., 2002) and cotton (44%, Kakani et al., 2005) are similar to the values obtained in the present study. Therefore, it is suggested that the response of *in vitro* pollen germination to temperature will be an accurate method to screen coconut varieties for high temperature tolerance. The cultivar San Ramon which had the highest T_{opt} (30 °C) also had the highest pollen germination percentage (30%). Similar pollen behaviour was observed in corn (Binelli et al., 1985) and snake melon (*Cucumis melo*) (Matlob and Kelly, 1973). Among the six coconut cultivars tested, Dwarf Green (DG) exhibited the widest temperature range ($T_{max} - T_{min} = 21.1$ °C) followed by TT (19.2 °C), the commercially-grown cultivar in Sri Lanka, indicating a wider temperature adaptability whilst Dwarf Brown (DB) had the smallest temperature range (17.7 °C) indicating a

narrower temperature adaptability. The average cardinal temperatures for pollen germination were 20.0 °C (T_{min}), 28.0 °C (T_{opt}) and 38.8 °C (T_{max}). Values reported for cotton were 14.0 °C (T_{min}), 31.0 °C (T_{opt}) and 43 °C (T_{max}) (Kakani et al., 2005) and for snake melon were 10 °C (T_{min}), 30 °C (T_{opt}) and 48 °C (T_{max}) (Matlob and Kelly, 1973). The varietal differences in pollen germination in this study could be due to the variation in their pollen carbohydrate concentrations (Firon et al., 2006). Under-utilization or unavailability of carbohydrates hinders pollen germination on exposure to high temperatures (Pressman et al., 2002). Experiments have been already started to verify the varietal differences in pollen carbohydrate concentrations and its role in determining the temperature tolerance of coconut pollen.

Pollen fertility is usually assessed in two ways; ie, percentage viability and percentage germination. However, in coconut, the pollen viability percentage which is always around 85 – 95% does not indicate the pollen fertility. Nevertheless, the pollen germination can vary in the range of 10% - 55% over the course of the year, possibly attributing to the variations in pollen carbohydrates which is the source of energy for germination. Therefore, in pollen germination studies of coconut, it is very important to mention the month or the season of pollen collection and take necessary precautions to minimize the effect of pollen collection period on achieving the desired objectives. As an example, in the present study, the pollen for experiment three was collected in the month of October and the highest % PG observed was about 30% whereas the pollen for experiment one was collected in November and the highest %PG observed for the same varieties was in the range of 45-55%.

Armendariz et al., (2006) compared the pollen fertility of micropropagated coconut plants with seed propagated palms, and pollen germination in both types of palms was reported to be 40%. Therefore, the results of the present study have to be further tested with pollen collected under different environment conditions before making a conclusion. Furthermore, the studies will be extended to establish the relationship between *in vitro* pollen germination (%PG) and fruit set and it will also be interesting to determine the minimum number of pollen germination required to have a successful fertilization and fruit set in coconut. The cardinal temperatures for pollen tube growth can also be determined, and a cumulative temperature response index, which uses all pollen parameters of interest to identify cultivar variability to high temperature, can be derived. Based on this index, it will be plausible to identify coconut cultivars tolerant, intermediate and sensitive to high temperature and used them in breeding programmes.

CONCLUSION

This is the first attempt to develop a screening technique for high temperature tolerance in coconut using *in vitro* pollen germination and pollen tube growth. The pollen parameters identified in the present study can be used as one of the parameters in the breeding programme to develop new hybrids for areas that are vulnerable to heat stress. In addition, the identified cardinal temperatures and response functions could be incorporated into process-based coconut models to increase prediction accuracy under current extreme and projected future climates.

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