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Differences in *in vitro* pollen germination and pollen tube growth of coconut (*Cocos nucifera* L.) cultivars in response to high temperature stress

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ABSTRACT

Temperature is a primary factor affecting the rate of plant development. Warmer temperatures expected with climate change and the potential for more extreme temperature events will impact plant productivity. Pollination is one of the most sensitive phenological stages to temperature extremes and in this study an *in vitro* pollen germination technique is used to screen coconut genotypes tolerant to high temperature. The pollen of twelve coconut genotypes comprising five talls (CCNT, FMST, LCT, PHOT and WCT), five dwarfs (CGD, COD, CRD, GBGD and MYD) and two hybrids (COD X WCT and MYD X WCT) were screened at different temperature levels from 10 to 50 °C at an interval of 2.5 °C. Cultivar variation existed for cardinal temperatures (T_{min}, T_{opt} and T_{max}) of pollen germination percentage and pollen tube growth. Mean cardinal temperatures calculated from the bilinear model for the 12 genotypes ranged from 23.5 °C to 29.5 °C, 9.7 °C to 16.5 °C and 40.1 °C to 43.9 °C for T_{opt}, T_{min} and T_{max}, respectively. In general tall, cultivars FMST, LCT, WCT, dwarf cultivar COD and hybrids showed better adaptability to high temperature while dwarf MYD was the least adaptable. At the metabolic level, high temperatures induced about 20% reduction in soluble protein content compared to optimal temperature in all the studied genotypes. There was an inverse relationship between superoxide dismutase activity and pollen germination percentage. Overall, there was wide variation in coconut cultivars for cardinal temperatures (T_{min}, T_{opt} and T_{max}) of pollen germination percentage and pollen tube growth. The genotypes with higher T_{max} for pollen germination and tube growth may be more tolerant to high temperature stress during flowering. Further studies are required to validate the results under *in vivo* condition; and also to understand the mechanism and factors that lead to pollen sterility in coconut.

1. Introduction

Rate of plant growth and development is dependent upon the temperature surrounding the plant and each species has a specific temperature range represented by a minimum, maximum, and optimum (Hatfield et al., 2011). The expected changes in global mean surface temperature over the next 30 to 50 years are predicted to be in the range of 1.4 to 2.6 °C based on scenario Representative Concentration Pathways (RCP 8.5) (IPCC, 2014). In future climates, heat waves or extreme temperature events are projected to become more intense, more frequent, and last longer than what is being currently observed (Meehl et al., 2007). Extreme temperature events may have short-term durations of a few days with temperature increases exceeding normal temperatures by over 5 °C. Such extreme temperature events can have large negative impacts on plant growth, development and yield (Prasad et al., 2017). Sexual reproduction in plants is more sensitive to increase in 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 stages of floral bud development (Prasad et al., 2017). Fruit set in field crops like cowpea (Ahmed and Hall, 1993), cotton (Reddy et al., 1997), groundnut (Prasad et al., 2003), field pea (Jiang et al., 2018) and tomato (Sato et al., 2002) and tree crops like sweet cherry (Hedhly et al., 2003), almond (Sorkheh et al., 2011), coconut (Ranasinghe et al., 2010) are sensitive to high temperatures. However, the information about the temperature effects in plantation crops like coconut is scanty and needs further attention.

Coconut is extensively cultivated in 12.3 million ha in 92 countries with an annual production of 67 billion nuts (APCC, 2016). Widely acclaimed as Kalpavriksha or tree of heaven, coconut provides food

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security and livelihood opportunity to 20 million people globally and 10 million people in India through cultivation, processing, marketing and trade related activities and thus it exerts profound influence on the rural economy. However, it is highly sensitive to both drought and high temperatures. Coconut palm experiences stress when exposed to excess radiation above 265 Wm^{-2}, temperature > 33 °C and vapor pressure deficit > 26 m bar (Kasturibai et al., 1988), aggravated by soil water deficit during the period. The duration of drought during initiation of inflorescence primordia, ovary development and button size nut, stages in that order, has greater influence on nut yield than other stages (Rajagopal et al., 1996).

Since coconut is a perennial crop, it has to cope with the impact of climate change during its life cycle. Unlike other crops, coconut flowers throughout the year. Hence, especially during summer it was found to be sensitive to high temperatures and significant yield reduction is observed (Ranasinghe et al., 2015). In cross pollinated plants like coconut, pollen has to remain detached from the plant, i.e. in the open environment for a long time without losing its viability. The time of anther dehiscence and releasing pollen depends on the prevailing environment but it is not clearly understood in coconut. The average germination percentage of coconut pollen grains was 23 to 40% (Ranasinghe et al., 2010; Armendariz et al., 2006). Pollen of plants is sensitive to minor fluctuations in atmospheric variables, especially temperature and can be used as an effective specimen to study whole plant stress tolerance. Also, pollen is essential in breeding programmes and maintenance of crop gene banks. So it is important to know about its viability under the scenario of global environmental change, particularly under high temperatures. Many studies on food grain crops showed that pollen development during various phases of microsporogenesis was sensitive to high temperature stress (Salem et al., 2007, Prasad et al., 2008, Jain et al., 2010). At the metabolic level, heat stress is known to alter the carbohydrate and protein level in pollen and the build-up of toxic by-products, such as ROS, which ultimately lead to pollen sterility and affect plant vegetative and reproductive development, with negative consequences on fruit set and yield (Bita and Gerats, 2013; Prasad et al., 2017; Djanaguiraman et al., 2017a,b). In many field crops, genotypes tolerant to elevated temperatures were selected through *in vitro* pollen germination studies (Kakani et al., 2002, 2005). However, information on the genetic variability in pollen germination and impacts of high temperature stress on pollen germination is limited in coconut. Thus, the objectives of this study were to (a) quantify the effect of temperature on pollen germination and pollen tube growth of 12 coconut cultivars *in vitro* and (b) study the biochemical changes take place in the pollen of selected cultivars and their relation with germination at different temperature.

2. Material and methods

2.1. Experimental location and coconut genotypes investigated

In vitro pollen germination was studied in 12 coconut genotypes belonging to talls, dwarfs and hybrids which were most widely cultivated and found to have wide adaptability to different environmental conditions (Table 1). The trees selected for this study were of 20 to 25 years old growing at a well-managed farm of ICAR-CPCRI (Indian Council of Agricultural Research – Central Plantation Crops Research Institute). The farm is located at 12° 18′ N latitude and 75°E longitude. It is about 10.7 m above mean sea level. In summer, this region has an average maximum temperature of 31.5 °C and minimum of 23.5 °C. The average relative humidity is about 88% and the region receives approximately 3400 mm rainfall annually. The soil is sandy loam type with 4.3–5.5 pH.

2.2. Pollen collection and growth medium

In vitro pollen germination experiment was conducted during December 2016 to March 2017. The maximum temperature during the period was around 32 °C and minimum temperature was 20 °C. It varies from season to season which has not been clearly studied in coconut. Hence, in order to obtain the pollen with high germination percentage for the experiment, a few trials were conducted before the start of the actual experiment. The day after inflorescence opening, spikelets with male flowers was collected from three inflorescences at 6.30, 8.30 and 10:30 amfor a period of 10 days in each of a dwarf (COD) and tall (WCT) variety. Pollen germination percentage of these flowers was recorded, allowing identification of the optimal time to measure pollen germination. We found a definite pattern on flower opening in the inflorescence, and the pollen viability was influenced by characteristics such as environmental humidity and temperature in confirmity with the earlier findings Ranasinghe et al., 2010; Nepi and Pacini, 1993). Once it was established, the procedure was used to evaluate the other genotypes.

To test the genotypic response to temperature, spikelets from the inflorescence (5 to 6 days after inflorescence opening) were collected at 8:30 amand immediately put in polythene bags and placed in an ice-box to avoid desiccation. In the laboratory, the male flowers collected were lightly tapped with a nylon brush so that they shed pollen and the same was collected on a wax coated paper. The pollen grains collected was transferred into a germination medium which consisted of 8% sucrose, and 0.01% boric acid in 100 mL deionized water (Karun et al., 2014). The media was solidified with 1% agar. Two mL of germinating media was spread on a glass slide which were then placed in Petri dishes lined with moist filter paper, thus maintaining a dish humidity of about 50% to avoid moisture accumulation and pollen rupture. The slides with germination medium were placed in the PID (Proportional-Integral-Derivative) microprocessor controlled BOD (Biological Oxygen Demand) incubator with respective temperature (between 10 and 50 °C at 2.5 °C intervals) for preconditioning (15 min) before sprinkling the pollen on the medium. Once the temperature equilibrated, pollens were uniformly sprinkled on the media and incubated for 2 h. Four slides per each plant at each temperature were used as replications.

2.3. In vitro pollen germination and pollen tube growth

Pollen germination and tube growth observations were made 2 h after incubation in all the treatments. Slides with germinated pollens were observed under 10× magnification objective of a Nikon Eclipse NI- U microscope (Nikon Corporation, Tokyo, Japan) paired with a Nikon DS – Ri1 camera. The photographs of the observed microscopic fields were taken at a resolution of 1920 × 1536 pixels and a scale of 0.61 µm/pixel with the help of the software, NIS Elements D version 4.3. Five microscopic fields per slide were photographed. Measurements of germination count were made using the 'counts' tool in the 'Measurement and Annotations' toolbar of NIS Elements D. A pollen grain was considered to have germinated when the length of the germinated pollen tube was equal to or longer than the diameter of the pollen (Kakani et al., 2002). Pollen tubes attained their maximum length in 2 h and beyond that time tubes began bursting. Hence, the tube length was measured at the end of 2 h incubation in all the treatments using the Polyline length measurement tool and expressed in micrometer. Percentage pollen germination was calculated using the formula:

$$\% \text{ Pollen germination} = \frac{\text{Number of germinated pollen}}{\text{Total number of pollen}} \times 100$$

The percentage pollen germination and pollen tube length data was averaged over all the microscopic fields per slide and all the slides per temperature treatment per palm. The replicated values on maximum pollen germination and tube length were analysed using the one-way ANOVA procedure (SAS, 1997).

Table 1
Name, origin, stature, plant height, breeding habit and special characters of 12 coconut cultivars evaluated for tolerance to high temperature.

Genotype	Origin	Stature	Height of the palm[a] (cm)	Breeding habit	Special character
WCT	India	Tall	1090	Cross pollinated	Water deficit stress tolerant, thick cuticle and parenchyma cells, low transpiration, high epicuticular wax deposition
PHOT	Philippines	Tall	1150	Cross pollinated	Less adapted to water deficit, thin cuticle, less deposition of epicuticular wax
CCNT	Vietnam	Tall	1230	Cross pollinated	Relatively tolerant to water deficit stress, good quality tender nut water
LCT	India	Tall	1110	Cross pollinated	Water deficit stress tolerant, thick cuticle and parenchyma cells, low transpiration, high epicuticular wax deposition
FMST	Malaysia	Tall	1042	Cross pollinated	Well adapted to water deficit stress
COD	India	Dwarf	642	Self pollinated	Sensitive to water deficit, good quality tender nut water
MYD	Malaysia	Dwarf	759	Self pollinated	Alternate bearing habit, Relatively more VAM colonization in relation to water deficit among dwarfs
CGD	India	Dwarf	540	Self pollinated	Slender trunk, susceptible to red palm weevil infestation, less adapted to water deficit stress, resistance to root (wilt) disease with high yield in diseased tracts
GBGD	India	Dwarf	579	Self pollinated	Less adapted to water deficit, good quality tender nut water and good general combiner
CRD	Africa	Dwarf	704	Self pollinated	High tender nut water content, large inflorescence among dwarfs
Chandra Sankara (COD X WCT)	India	Semi tall	985	Cross pollinated	Relatively sensitive to water deficit stress, precocious bearing, high yield under irrigation and good management conditions.
Kalpa Samrudhi (MYD X WCT)	India	Semi tall	960	Cross pollinated	Relatively better tolerant to moisture stress, precocious bearing, high nitrogen use efficiency.

[a] Height was measured from the base of the trunk to top of the canopy in 25 years old palms.

2.4. Curve fitting and analysis

Maximum pollen germination percentage and pollen tube length recorded after 2 h of incubation, at each temperature, were analysed using linear and nonlinear regression techniques to quantify developmental responses to temperature as described by Kakani et al. (2005). Quadratic (Yan and Wallace, 1998), cubic or higher order polynomial (Tollenaar et al., 1979) and modified broken-stick or bilinear (Omanga et al., 1995) equations were applied to data and examined to determine the best fit model.

The modified bilinear equation (eqn 1) provided the highest R^2 value and smallest root mean squared deviation (RMSD) for both pollen germination and pollen tube length and was used to estimate cardinal temperatures viz. minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}), for pollen germination and pollen tube length of all cultivars (Kakani et al., 2002). Analyses were performed using the PROC NLIN procedure in SAS 9.3 (SAS, 1997). A modified Newton–Gauss iterative method was used to determine T_{opt} based on the lowest RMSD values between observed and predicted values. Values of T_{min} and T_{max} were estimated using parameters derived from the modified bilinear equations (eqns 2 and 3).

$$\text{Pollen germination (\%) or pollen tube length} = a + [b_1 (T - T_{opt})] + [b_2 (ABS (T_{opt} - T))] \quad (1)$$

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

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

where a, b_1 and b_2 are equation constants, T the various temperatures at which germination and tube growth were studied, and T_{opt} the optimum temperature for germination or pollen tube growth.

2.5. Cluster analysis

Cardinal temperatures (T_{min}, temperature below which there was no pollen germination; T_{max}, temperature above which there was no pollen germination; $T_{opt,}$ temperature at which maximum pollen germination was observed) and the maximum pollen germination (%) derived from the empirical modeling could easily differentiate the genotypes. Clustering was done using the parameters viz. T_{min}, T_{opt}, T_{max} and estimated maximum pollen germination (a_pg) to group the large number of genotypes into smaller number of clusters (clustering) in order to study the similarities and diversities among the genotypes.

2.6. Determination of biochemical constituents

Cultivars identified as tolerant (COD, LCT and WCT) plus the susceptible MYD cultivar from the *in vitro* pollen germination study were used for biochemical analysis. Pollen samples (0.1 g in 1 mL medium) were incubated in the preconditioned medium consisted of 8% sucrose, and 0.01% boric acid at different temperature (10 to 50 °C at an interval of 5 °C for 2 h), and then homogenized in a PRO250 Homogenizer with 80% ethanol. This homogenate was centrifuged at 5000 rpm at 25 °C for 10 min and the extract was repeated twice. Supernatant collected was pooled and used for the analysis. It was homogenized with 0.2 M sodium phosphate buffer (4 mL), and the homogenate was centrifuged at 10000 rpm at 4 °C for 15 min. Supernatants were collected, aliquots in multiple small volumes and immediately stored at −20 °C before use.

Total sugar present in the above aliquots was estimated by phenol sulphuric acid method (DuBois et al., 1956) and reducing sugar was estimated using the method of Nelson-Somogyi (Somogyi, 1952). The concentration of both total and reducing sugar present in the sample was calculated from the calibration curve developed using glucose as standard. Free amino acid in pollen was estimated using the Ninhydrin method (Moore and Stein, 1948). The protein content in pollen was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin as a standard. Super oxide dismutase (SOD) specific activity was assayed by following the method of Beauchamp and Fridovich (1971), and one unit of SOD specific activity was calculated as the 50% reduction of the blue color formed by NBT 30 min^{-1} mg $protein^{-1}$. All the experiments were replicated thrice and the results presented as mean ± SD.

2.7. Statistical analysis

In vitro pollen germination and tube length response to temperature was studied in six plants of each cultivar and for each temperature there were four slides. Thus there were observations from 24 slides for each temperature of a cultivar which was used for curve fitting to determine the cardinal temperatures. Curve fitting analysis and clustering was done using the SAS software version 9.3 (SAS, 1997). Cultivar comparison, temperature response and biochemical comparisons were analysed using ANOVA proceeds in SAS 9.3 and treatment means were separated using Duncan's multiple range tests.

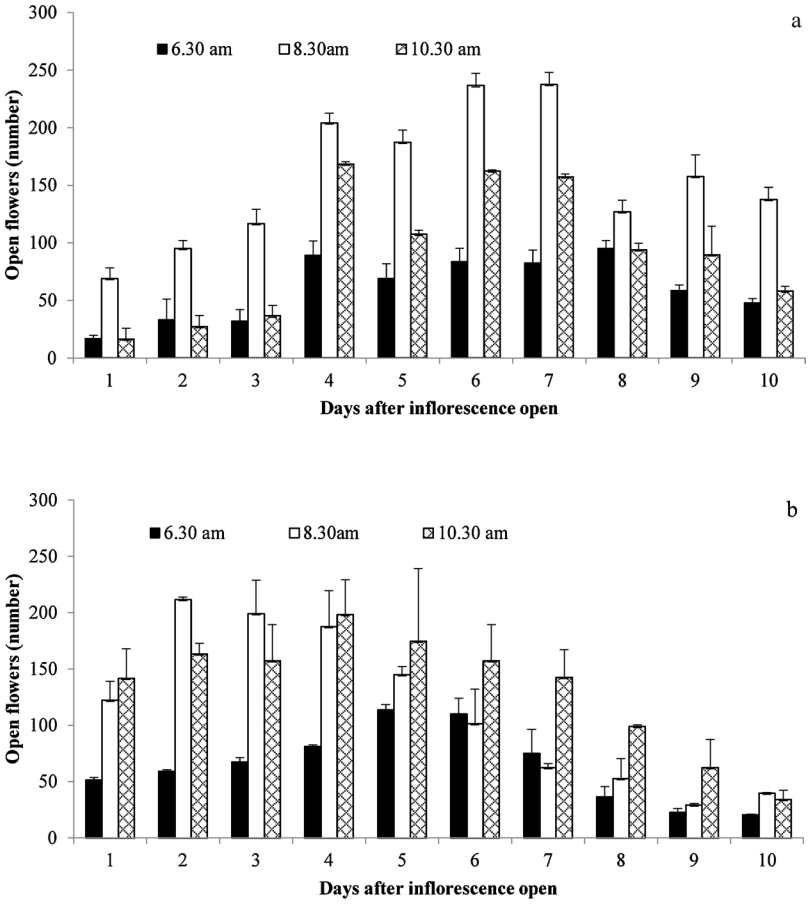

Fig. 1. Flower opening pattern during day from the date of inflorescence opening for a period of 10 days of a (a) tall variety WCT and a (b) dwarf variety COD. Flowers opened at 6.30, 8.30 and 10:30 am were collected and counted. Bars indicate the SD between the replications (n = 3). CD (P > 0.05%) values across flower opening time was 6.34 and 13.36 and across days after inflorescence opening was 11.58 and 24.41 for WCT and COD, respectively.

3. Results

3.1. Pattern of male flower opening and pollen germination

In order to find the time of male flower opening and pollen viability during the relatively dry month of January, open flowers and their pollen were collected from two contrasting genotypes of dwarf (COD) and tall (WCT). From Fig. 1(a) and (b) it is clear that the flower-opening pattern varied quite distinctly with genotype. In WCT, a large number of open flowers was seen around 4 to 7 days while in COD it was between 2 and 5 days after opening of the inflorescence. In both the varieties however, significantly high number of male flowers open at 8.30 am compared to either 6:30 am or 10:30 a.m. However, in COD beyond 5 days of inflorescence opening high number of open flowers were seen between 8.30 and 10:30 am The germination of pollen also varied significantly in those male flowers opened at different time of the day. As can be observed from Fig. 2, germination was high in the flowers opened at 8:30 am than those opened at 6:30 am and 10:30 am in both the genotypes COD (63%) and WCT (68%).

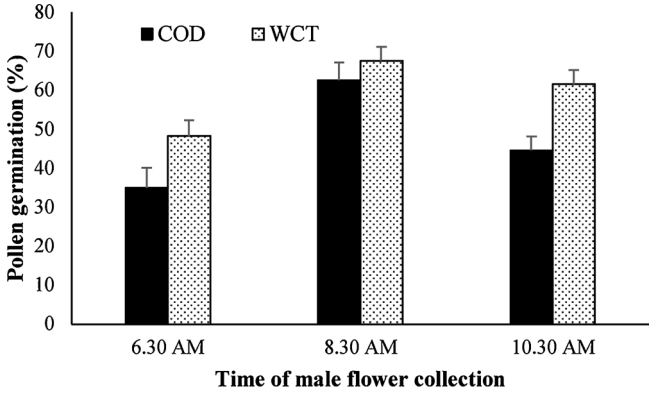

Fig. 2. Germination percentage of fresh pollens from the open flowers collected at 6.30, 8.30 and 10:30 am of the coconut variety COD and WCT. Error bars indicate the SD between the replications (n = 3).

3.2. In vitro pollen germination

Pollen grains started germinating in about 10 min on contact with the germination medium. Fig. 3 showed the variation for pollen germination of two genotypes *viz.* tall (WCT) and dwarf (MYD) in response to different temperature for clarity. Germination was high in WCT and showed wider adaptability to temperature compared to MYD. The modified bilinear equation described the response of pollen germination well, with an average R^2 for all genotypes of 96.3% (range 91–99%). The observed cultivar differences for both germination percentage and cardinal temperature were significant (Table 2). The maximum percentage germination ranged from 27.3% (MYD) to 65.3% (MYD X WCT) with a mean of 48.5%. The cardinal temperatures also differed greatly among genotypes (Table 2). Values of T_{min} ranged from 9·7 °C (LCT) to 16·5 °C (GBGD), T_{opt} from 23.5 °C (COD) to 29.5 °C (WCT) and T_{max} from 40.1 °C (CCNT) to 43.9 °C (COD X WCT). Genotypic variation in T_{max} was less than for $T_{min.}$

3.3. Pollen tube length

The response of maximum pollen tube growth to temperature was similar to that of pollen germination and was well defined using the modified bilinear model (Table 3). There was significant variation among genotypes in maximum pollen tube length that ranged from 396 µm (CGD) to 575 µm (GBGD) with a mean of 489 µm. The mean

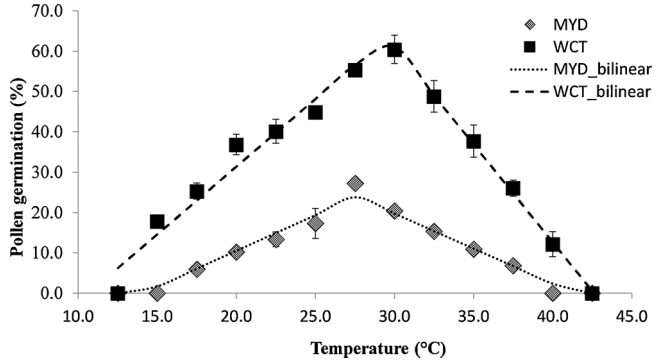

Fig. 3. Pollen germination in response to temperature and their fitted lines based on modified bilinear model of two coconut cultivars, a dwarf (MYD) and a tall (WCT). Error bars indicate the standard deviation.

values of T_{min}, T_{opt} and T_{max} for pollen tube growth were 12.8, 27.5 and 43.0 °C. The range of values for T_{min} and T_{max} were 9.7 (FMST) to 17.7 °C (GBGD) and 40.0 (CCNT) to 48.0 °C (COD), respectively, similar to those for pollen germination. The mean T_{opt} for maximum pollen tube length, however, was 1 °C higher than that for pollen germination (27.5 compared with 26.5 °C). Cultivars differed significantly in pollen tube length at optimum temperatures. The T_{opt} ranged from 26.2 °C to 30.2 °C and it was high for CCNT, CRD, FMST and GBGD compared to COD and COD x WCT and WCT.

3.4. Pollen germination of contrasts

Among the cultivars, WCT from talls and COD from dwarfs showed high pollen germination and pollen tube length. However, their response to temperature varied widely at high and low temperature from the optimum (Fig. 4a). Temperature at lower than optimum (between 15 °C–25 °C) COD exhibited better percentage pollen germination and tube length than WCT while at higher temperature than optimum (between 27.5 to 40 °C) WCT showed higher pollen germination and tube length. Similar variability was observed even amongst the genotypes of the same group. On comparing both dwarfs COD and GBGD it was clear that, relative performance of COD was good at lower temperature between 15 °C–27.5 °C (Fig. 4b). However at higher temperature beyond optimum GBGD showed better pollen germination and tube growth.

3.5. Clustering of genotypes

A dendrogram drawn based on variables like T_{opt}, T_{min} and T_{max} for pollen germination and estimated maximum value of pollen germination ('a_pg') exhibited differential similarities among the genotypes and accordingly genotypes under study were grouped into 3 clusters. As depicted in Fig. 5, the first cluster includes COD, FMST, LCT, WCT, COD X WCT and MYD X WCT. The second cluster includes CCNT, CGD, CRD, GBGD and PHOT and the third cluster consisted of MYD alone. The optimum temperature for pollen germination was highest for cluster 3 (27.6 °C) followed by 26.2 °C for both 1st and 2nd cluster (Table 4). The T_{min} value was maximum for cluster 2 (15.2 °C) followed by cluster 3 (15 °C) and was least for cluster 1 (10.9 °C), whereas the T_{max} was highest for cluster 1 (43 °C) followed by cluster 2 (41.6 °C) and was least for cluster 3 (40.4 °C). Similarly the estimated maximum value for pollen germination was highest (62.5%) for cluster 1 followed by cluster 2 (47.8%) and was least for cluster 3 (25.1%).

3.6. Biochemical changes in pollen

Total sugar content of germinating pollen grains showed a significant decline with increasing temperature in all the genotypes except MYD where it was non-significant (Table 5). It showed a steady decline with increase in temperature up to T_{opt} (30 °C) beyond which there was no definite pattern. At 30 °C it was the highest for MYD (720 mg/g) followed by LCT (698 mg/g), WCT (685 mg/g) and COD (624 mg/g). In all the cultivars studied, total sugar content negatively correlated with germination percent. Unlike total sugar, reducing sugar content significantly increased with increase in temperature in all the genotypes. It was less in control (6.70, 20.1, 16.8 and 25.3 mg/g pollen for WCT, LCT, COD and MYD, respectively) which gradually increased and reached the maximum at 50 °C (349, 266, 392 and 322 mg/g pollen for WCT, LCT, COD and MYD, respectively). Significant positive correlation was observed between the germination percent and the reducing sugar content among the cultivars studied.

Similar to total sugar, amino acid content too did not show significant response to temperature in MYD, but in all other genotypes, it was significantly high at T_{opt}. As such MYD had high amino acid content and at 30 °C it was 35.0 mg/g in comparison to 33.5, 30.7 and 31.1 for COD, LCT and WCT respectively (Table 5). Beyond 30 °C amino acid steeply declined in WCT followed by LCT and COD while the decline was gradual in MYD. Protein content in the pollen almost followed a similar pattern as that of amino acids. The content was minimal at 10 °C, increased gradually and reached its maximum at 25 °C to 35 °C. It peaked at 25 °C for MYD (185 mg/g), 30 °C for WCT (151 mg/g) and

Table 2
Maximum pollen germination percentage, modified bilinear equation constants and cardinal temperatures for pollen germination of 12 coconut cultivars in response to temperature.

Genotype	Maximum pollen Germination (%)	Equation constants				Cardinal temperature		
		a	b_1	b_2	R^2	T_{opt}	T_{min}	T_{max}
COD	56.1 ± 3.07	61.3	1.05	−4.15	0.98	23.5	11.7	43.3
CRD	39.2 ± 4.93	41.4	0.45	−3.14	0.99	26.9	15.4	42.3
MYD	27.3 ± 0.81	25.1	0.00	−1.98	0.97	27.7	15.0	40.4
GBGD	45.8 ± 1.45	49.4	0.15	−3.73	0.98	29.3	16.5	43.0
CGD	41.2 ± 1.15	39.8	0.12	−2.69	0.94	26.3	12.2	41.8
WCT	60.4 ± 5.97	63.4	−0.74	−4.10	0.98	29.5	10.6	42.6
LCT	49.2 ± 1.65	55.1	−0.20	−3.35	0.91	27.2	9.70	42.7
FMST	50.8 ± 6.48	56.6	−0.12	−3.54	0.96	26.9	10.4	42.4
PHOT	43.4 ± 3.06	51.8	1.59	−4.51	0.95	23.8	15.3	41.5
CCNT	45.2 ± 2.92	49.8	0.10	−3.91	0.98	27.0	14.5	40.1
MYD X WCT	65.3 ± 2.10	71.1	0.60	−4.69	0.98	25.7	12.3	43.1
COD X WCT	58.2 ± 0.73	67.7	0.67	−4.19	0.93	24.7	10.8	43.9
Mean	48.5					26.5	12.9	42.3

Values of maximum pollen germination% is given as mean ± standard error; a-estimated maximum pollen germination; b_1, b_2- equation constant; T_{min}, T_{opt} & T_{max} – cardinal temperature minimum, optimum and maximum, respectively.

Table 3
Maximum pollen tube length (PTL), modified bilinear equation constants, and cardinal temperatures for pollen tube length of 12 coconut cultivars in response to temperature.

Genotype	Maximum PTL (μm)	Equation constants				Cardinal temperature		
		a	b_1	b_2	R^2	T_{opt}	T_{min}	T_{max}
COD	408 ± 25.4	480	4.05	−26.21	0.83	26.3	10.4	48.0
CRD	464 ± 63.7	525	16.45	−45.22	0.95	30.2	15.0	41.7
MYD	436 ± 118	506	3.16	−35.32	0.89	26.8	13.7	42.6
GBGD	575 ± 6.90	577	9.52	−46.32	0.97	28.1	17.7	43.7
CGD	396 ± 90.4	431	0.74	−28.43	0.92	26.9	12.2	42.5
WCT	554 ± 12.4	667	2.67	−41.83	0.93	26.2	11.2	43.3
LCT	527 ± 9.80	589	0.60	−37.17	0.96	26.3	10.8	42.4
FMST	415 ± 21.0	454	−2.63	−27.51	0.95	27.9	9.7	43.0
PHOT	555 ± 48.6	646	5.89	−47.14	0.93	26.4	14.2	42.0
CCNT	485 ± 36.0	528	−2.82	−42.81	0.98	28.4	15.2	40.0
MYD X WCT	516 ± 8.42	567	0.73	−36.39	0.96	27.1	11.9	43.0
COD X WCT	540 ± 16.4	588	1.54	−36.70	0.98	26.7	11.3	43.5
Mean	489					27.5	12.8	43.0

PTL- pollen tube length; Values of PTL is given as mean ± standard error; a-estimated maximum pollen tube length; b_1, b_2- equation constant; T_{min}, T_{opt} & T_{max} – cardinal temperature minimum, optimum and maximum, respectively.

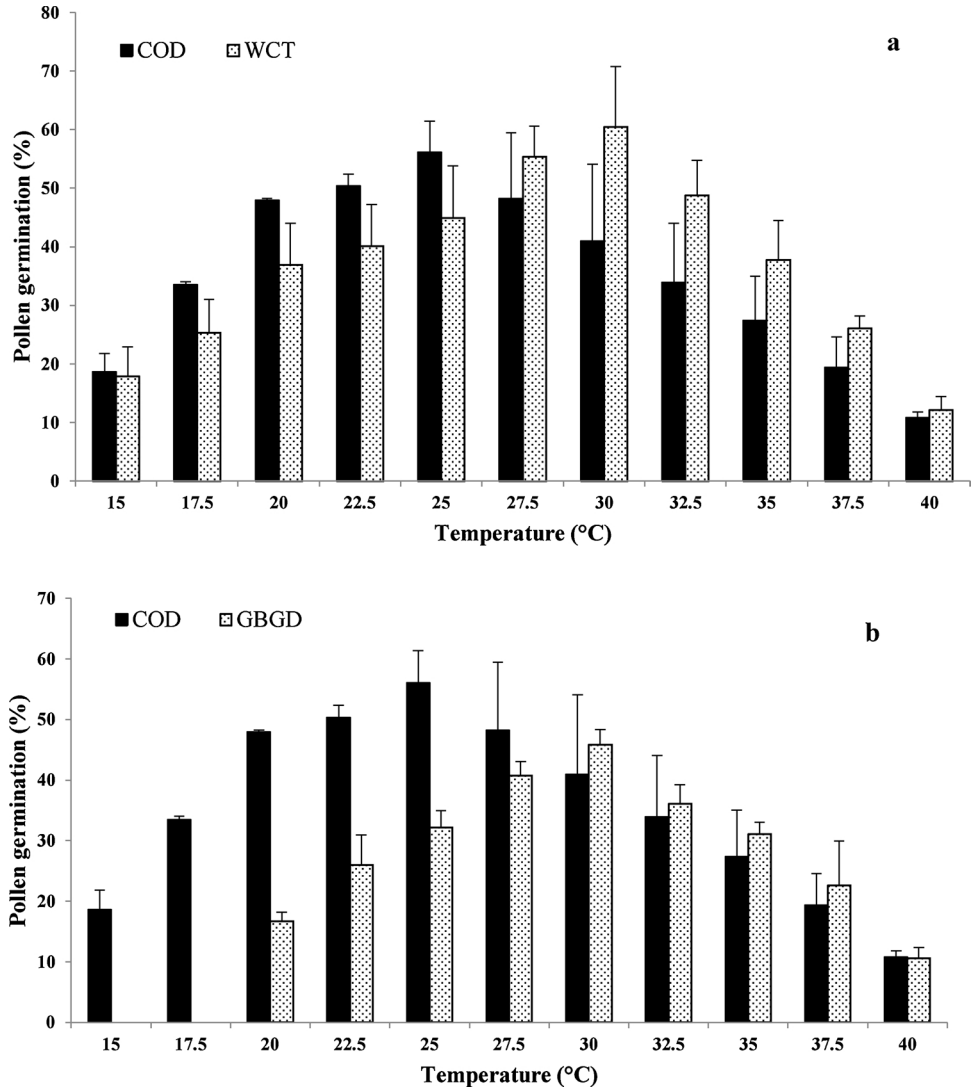

Fig. 4. Pollen germination percentage of contrasting genotypes (a) COD and WCT and (b) COD and GBGD at different temperatures. Error bars indicate the SD between the replications (n = 3). CD (P = 0.05%) values between the genotypes was (a) 4.3 and (b) was 7.6.

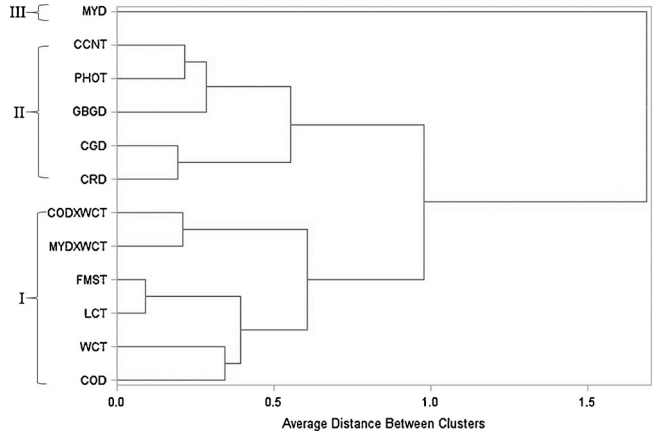

Fig. 5. Clustering of coconut genotypes studied for pollen germination at different temperature.

Table 4
Cluster means of various characters.

Characters	Cluster I	Cluster II	Cluster III
T_{opt}	26.2	26.2	27.6
T_{min}	10.9	15.2	15.0
T_{max}	43.0	41.6	40.4
a_pg	62.5	47.8	25.1

T_{min}, T_{opt} & T_{max} – cardinal temperature minimum, optimum and maximum, respectively; a_pg – estimated maximum value of pollen germination.

LCT (165 mg/g) and 35 °C for COD (134 mg/g) (Table 5). Significant decline in protein content was observed at 40 °C when most of the cultivars had ceased their germination. The decline from the peak was steep for MYD (155 mg/g), while it was marginal for WCT (125 mg/g), LCT (153 mg/g), and COD (126 mg/g). Amino acid had significant positive correlation with pollen germination for four cultivars studied, while protein content in LCT and WCT had significant positive correlation with pollen germination. Contrary to the amino acids and protein content, the specific activity of SOD was significantly low at 30 °C in all the cultivars except COD in which it was non-significant. Beyond 30 °C it started increasing and at 40 °C it was highest for COD (20.2 unit), followed by MYD (15.2 unit), LCT (10.2 unit) and the least for WCT (6.18 unit).

4. Discussion

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. In a tall cultivar WCT and a dwarf cultivar COD used in this study, open male flowers could be seen since the first day of opening of inflorescence during the months of December to March. However, peak open flowers were seen between 1 and 5 days in COD while in WCT it was between 4 and 7 days of opening of an inflorescence. In dwarfs like COD, being mostly self-pollinated, the male flowers are known to open even before the spathe is completely open and the inflorescence is exposed (Menon and Pandalai, 1958). Open male flowers do not remain on an inflorescence for more than one day, generally open in the early hours of the day and then shed the same evening. In this study, most of the male flowers opened between 8:30 amand 10:30 am which is in agreement with the earlier findings of Menon and Pandalai (1958). We also observed high pollen germination in those flowers which were opened around 8:30 am Hence, in our studies we used pollens collected from male flowers opened at 8:30 ambetween 4 and 6 days after inflorescence emergence.

In this study, coconut cultivars selected from different origins and stature (tall and dwarf) showed wide variability in *in vitro* pollen germination and tube growth. The average germination observed was 48%, which is quite high compared to the previous reports of 23% for pollen collected during October/November periods (Ranasinghe et al., 2010) or 40% by Armendariz et al. (2006). In earlier studies, sun-dried pollen grains were often pooled, whereas in this study we used fresh pollen grains collected at 8:30 amwhich could have contributed to high germination percentage. Most of the dwarfs (CGD, CRD, GBGD and MYD) except COD had low germination while talls and hybrids had high germination percentage. Indian origin cultivars WCT, COD had high germination percentage while it was low for MYD which is a Malaysian origin. After germination, pollen tube started growing within few minutes and by two hours maximum growth was seen. Beyond that it started bursting (Fig. 6). Pollen tube lengths similar to those recorded in the present study (Table 3; average 489 um) were reported for coconut when pollen was grown on artificial media (Ranasinghe et al., 2010). Therefore, the observed differences in pollen germination and pollen tube length in the present study were a reflection of cultivar variability.

In this study, the response of coconut cultivars to temperature was studied from 10 to 50 °C at an interval of 2.5 °C and the cultivar differences for cardinal temperatures were estimated. The modified bilinear model best described the response of pollen germination to temperature (Tables 2 and 3). The average cardinal temperatures for

Table 5
Total and reducing sugar, amino acids and protein content in germinating pollen grains of four coconut genotypes incubated at different temperature.

Treatment	WCT				LCT				COD				MYD			
	TS	RS	FAA	Protein	TS	RS	FAA	Protein	TS	RS	FAA	Protein	TS	RS	FAA	Protein
Control	741.4^{a}	6.7^{e}	25.8^{ab}	106.4^{d}	848.0^{a}	20.1^{f}	27.65	125.0^{f}	716.4^{a}	16.8^{f}	21.65^{d}	124.3^{ab}	738.4	25.3^{f}	24.6	139.5^{cdb}
10 °C	683.2^{ab}	17.8^{e}	29.0^{ab}	111.4^{cd}	804.7^{ab}	34.2^{f}	25.73	139.2^{de}	709.7^{a}	34.8^{e}	26.13^{cdb}	121.5^{ab}	837.9	39.5^{ef}	26.9	149.6^{cb}
15 °C	697.7^{ab}	27.6^{e}	27.1^{ab}	120.7^{cdb}	742.0^{cb}	61.4^{fe}	25.73	145.2^{cdeb}	649.9^{cab}	61.2^{de}	23.84^{cd}	121.7^{ab}	679.4	42.4^{efd}	24.0	168.6^{ab}
20 °C	695.1^{ab}	61.8^{ed}	26.9^{ab}	129.7^{abcd}	712.3^{c}	102.6^{de}	28.33	146.8^{cdb}	683.2^{ab}	73.0^{de}	28.79^{cab}	119.1^{cb}	778.4	50.6^{efd}	31.4	161.8^{cab}
25 °C	693.8^{ab}	120.7^{cd}	28.2^{ab}	136.1^{cab}	701.3^{c}	111.9^{d}	30.08	155.7^{ab}	627.9^{cab}	124.3^{d}	31.67^{a}	130.7^{ab}	627.7	100.3^{cd}	30.5	185.1^{a}
30 °C	685.2^{ab}	148.6^{c}	31.1^{a}	151.6^{a}	698.1^{c}	127.7^{d}	30.65	165.6^{a}	624.3^{cab}	208.7^{c}	33.47^{a}	128.5^{ab}	720.1	92.7^{ecd}	35.0	171.1^{ab}
35 °C	681.2^{ab}	170.7^{c}	25.5^{ab}	143.4^{ab}	686.1^{c}	177.6^{c}	29.14	163.6^{a}	572.4^{cdb}	256.9^{c}	30.07^{ab}	134.0^{a}	579.3	143.7^{c}	27.4	159.6^{cab}
40 °C	657.0^{b}	247.2^{b}	22.2^{cb}	127.5^{cadb}	673.1^{c}	221.2^{b}	26.12	152.8^{cab}	594.4^{cadb}	327.5^{ab}	26.23^{cdb}	125.5^{ab}	669.7	135.1^{c}	25.3	154.8^{cab}
45 °C	648.0^{cb}	278.3^{b}	17.3^{c}	120.0^{cdb}	671.2^{c}	235.4^{ab}	24.31	142.1^{cde}	522.3^{cd}	322.2^{b}	23.02^{d}	117.1^{cb}	535.0	245.8^{b}	26.0	132.9^{cd}
50 °C	582.1^{c}	348.7^{a}	16.9^{c}	116.1^{cd}	581.1^{d}	266.3^{a}	22.91	132.9^{fe}	481.7^{d}	391.8^{a}	21.48^{d}	107.0^{c}	625.4	321.6^{a}	26.3	116.9^{d}
CD	70.20	66.55	7.61	24.43	80.54	42.09	NS	12.09	117.51	64.70	4.64	12.42	NS	54.20	NS	29.59

All values are expressed as mg per g pollen. CD- critical difference at 5% level of significance; Different letters in each column after the mean value are significantly different at 5% level of significance according to DMRT. TS – total sugar; RS – reducing sugar; FAA – free amino acids. The values obtained for total sugar includes the sucrose in the medium.

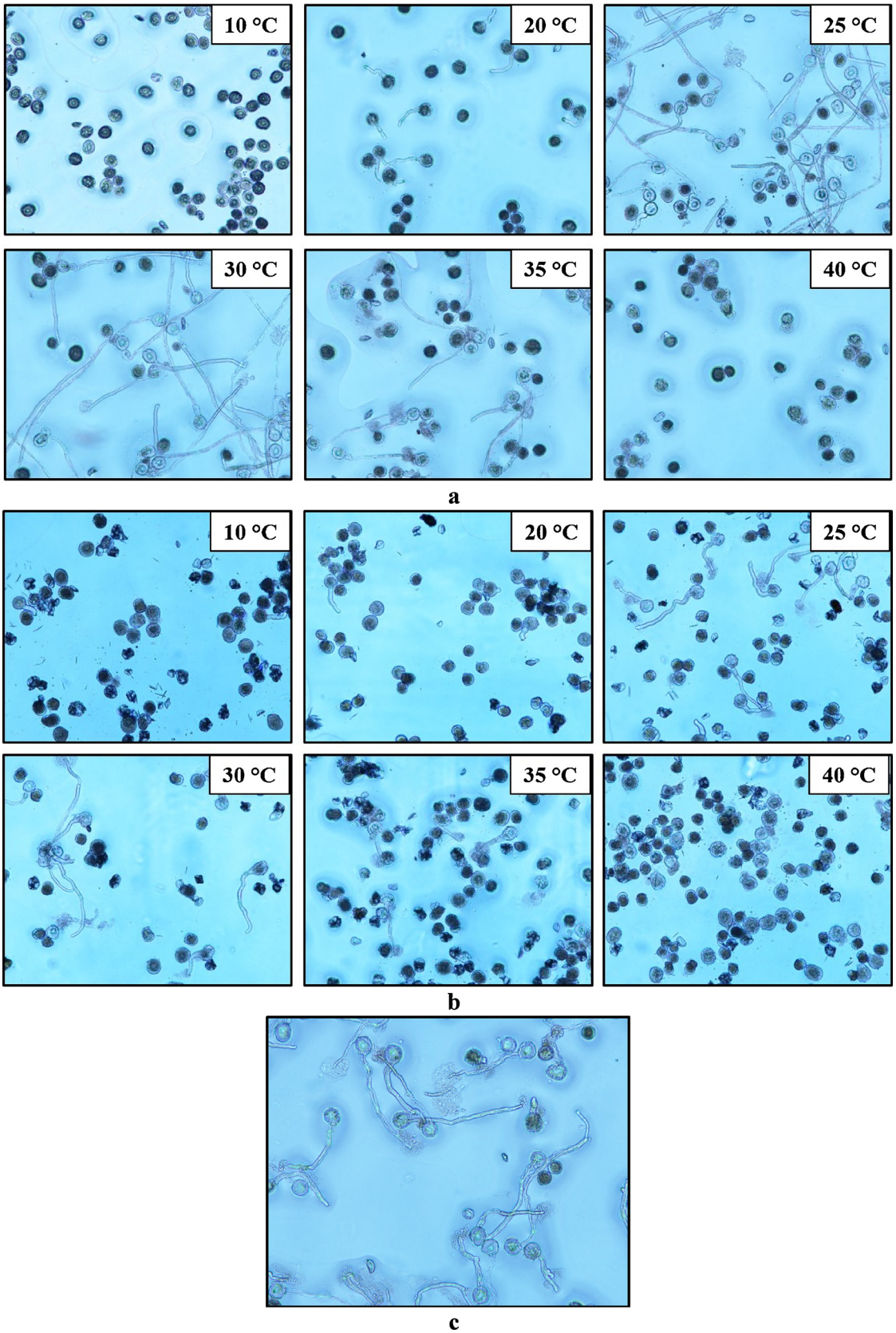

Fig. 6. Germinated pollen grains in the medium under *in vitro* condition. Pollen grains were dusted on the preconditioned medium and incubated for 2 h at different temperatures. Pollen germination and pollen tube growth of a tolerant variety WCT (6a) and a susceptible variety MYD (6b). 6c. displays the phenomena of bursting of pollen tubes incubated at 25 °C for 3 h.

pollen germination were 12.9 °C (T_{min}), 26.5 °C (T_{opt}) and 42.3 °C (T_{max}). The T_{opt} recorded for pollen germination was similar to the optimum temperature requirement for plant growth and development 27 ± 3 °C (Child, 1974). However, it is lower than other field crops like cotton 31.8 °C (Kakani et al., 2005), peanut 32 °C (Kakani et al., 2002), snake melon 30 °C (Matlob and Kelly, 1973). T_{opt} for coconut cultivars ranged from 23.5 in COD to 29.5 °C in WCT. However, the differences in T_{opt} did not reflect the tolerance or susceptibility of a cultivar to high temperatures because the cultivars which had a higher optimum temperature did not always have a higher temperature maximum or vice versa. For example COD which had low T_{opt} had high T_{max} while WCT with high T_{opt} had relatively low T_{max}. However, there was better relation between T_{opt} and T_{min}. Most of the dwarfs had high T_{min} except COD, which had relatively low T_{min}. COD displayed better germination and tube growth at lower than T_{opt} while WCT and GBGD performed better under higher temperature than T_{opt}. Therefore, WCT and GBGD are more adaptable to high temperature stress compared to COD.

Most of the Indian origin cultivars COD, GBGD, LCT, WCT, COD x WCT, MYDxWCT along with FMST (Malaysian origin) had high T_{max} both for pollen germination and pollen viability. Most of these genotypes except COD are known to be tolerant to abiotic stresses like water deficit stress (Rajagopal et al., 1990). In *Brassica napus* it was suggested that reduced pollen germination under high temperature is the major cause of low pollen fertility (Young et al., 2004). Prasad et al. (1999) in peanuts and Aloni et al. (2001) in bell pepper established a high correlation between *in vitro* pollen germination and fruit-set/seed-set under high-temperature conditions; this suggests that cultivar differences observed in pollen germination in our study could be a useful tool for testing cultivar tolerance to high temperature. Therefore, the ability of pollen to germinate and grow well at temperatures above 27 °C could be used as a tool to identify high-temperature tolerance in coconut cultivars.

Based on T_{opt}, T_{min}, and T_{max} and estimated maximum value for germination genotypes were grouped into three clusters. Clusters 1 had relatively high T_{opt} and low T_{min} and high T_{max} and thus have wider adaptability (WCT, LCT, COD, FMST and hybrids) and these genotypes also had high germination. Cluster 2 is sensitive to T_{min} but relatively tolerant to T_{max} (PHOT, CCNT, GBGD, CGD and CRD) and thus moderately adaptable to high temperature. Cluster 3 on the other hand had high T_{min} and low T_{max}, poor germination (MYD) thus less adaptable to both low and high temperature.

In order to ascertain the factors responsible for sensitivity of pollen germination to high temperature, metabolites likes total sugar, reducing sugar, amino acid and protein content along with activity of SOD, a free radical scavenging enzyme was measured in germinating pollen of contrasting cultivars to high temperature [tolerant (WCT, LCT and COD) and sensitive (MYD)]. The reduction in the total sugar content in the pollen was due to the utilization of same during the germination process. It steadily declined in WCT, LCT and COD while no significant decline was seen in MYD suggesting underutilization of carbohydrates hindered pollen germination on exposure to high temperatures in MYD (Pressman et al., 2002). The reducing sugar content showed higher value at higher temperature, and that could be due to the result of inversion (hydrolysis of sucrose into glucose and fructose). Decreased rate of inversion and the reduced germination rate at lower temperature (15 °C to 30 °C) could be the reason for reduction in level of reducing sugar. At higher temperature, the rate of inversion could be more than the utilization, and thus the reducing sugar level increased. Among the genotypes, at lower temperature, COD had better inversion rate compared to other genotypes studied and it reflected in higher germination, while in case of MYD the inversion rate is less, which affected the germination rate. MYD also had low reducing sugar content when compared to COD at high temperature (40 °C to 50 °C). It was reported that during germination, pollen grains depend mostly on simple sugar as metabolic substrate for their germination (Stanley, 1971). In case of tomato plants, reducing sugar was predominating in pollen followed by maltosaccharides, and sucrose was at very low concentration (Carrizo et al., 2009, 2010). Reduction in total sugar content and increase in reducing sugar content in the coconut pollens indicated that the sucrose is the major carbohydrate sources utilized during the pollen germination.

Palm pollen grains contained 8 essential amino acids and 9 non-essential amino acids (Hassan, 2011). From the present study it was clear that the amino acid content was high (30–35 mg/g where there was high germination and pollen tube growth and was lower in the temperature treatments where there was less germination. Increase in free amino acid content in the coconut pollen germinated under different temperature indicated that *de novo* synthesis of amino acids take place during the pollen germination. Amino acid content followed the same trend as that of pollen germination in all the cultivars except MYD. MYD had relatively high amino acid at high temperature (beyond 30 °C), this accumulation might not be *de novo* synthesis but as a consequence of breakdown of proteins at high temperature. From the data it is clear that in MYD beyond 30 °C there was a decline in protein content (reduced from 171.1 mg at 30 °C to 116.9 mg at 50 °C) while in other cultivars it was a steady decline. Thus, it is evident that MYD at high temperature lacks the ability to synthesize protein which could have limited germination of pollen beyond the optimum temperature. Protein in the pollen becomes apparent when the pollen grain starts to germinate. Soluble protein was initially absent and begins to increase rapidly as the pollen germinates (Stanley and Linskens, 1965; Linskens and Schrauwen, 1969). This agrees with the results obtained in the present study that the protein in coconut pollen showed less value during higher and lower temperature where germination is less, compared to that of temperatures where germination was high. Soluble proteins are one of the key factors essential for pollen development and germination, and their disruption due to temperature stress may lead to loss of their activity (Plif, 1981; Song et al., 1999). Earlier reports also suggested under high temperature stress the rate of synthesis of protein is affected significantly (Ruberti and Brandizzi, 2014). Tang et al. (2008) also reported that high temperature inducted reduction in soluble protein content in rice pollen grains. The increased activity of SOD at low and high temperature indicated that the pollen grains had experienced stress, which is in conformity with the activity observed in other crops under temperature stress (Kaushal et al., 2011; Toscano et al., 2016). Both dwarf cultivars COD and MYD had strong SOD activity but were not sufficient to prevent the damage caused by the stress to the process of protein synthesis.

From the study it is clear that there is wide variability in coconut cultivars for *in vitro* pollen germination in response to temperature. Cultivars WCT, LCT, COD and hybrids showed better adaptability than the dwarf cultivar MYD which is highly sensitive to temperature. As it was demonstrated in field crops, it is a simple technique to screen cultivars. In coconut, it is highly relevant to screen the cultivars for high temperature tolerance that is imminent under climate change. Coconut being perennial and a large tree it is very difficult to impose the high temperature treatment even for study purpose. However, the finding from the *in vitro* studies need to be tested under *in vivo* at different agroclimatic conditions (with temperature difference) by effecting artificial pollination using the selected tolerant pollens. Similarly further in depth studies are needed to study the biochemical parameters like lack of protein inhibiting germination in high temperature.

5. Conclusions

There was genetic diversity in coconut cultivars for cardinal temperatures (T_{min}, T_{opt} and T_{max}) for pollen germination percentage and pollen tube growth. The genotypes with higher T_{max} for pollen germination and tube growth may be more tolerant to high temperature stress during flowering. Further studies are required to validate the results under *in vivo* condition. If confirmed this provides a method to

screening germplasm for high temperature tolerance in coconut. Targeted breeding and incorporation of high temperature tolerance during flowering will be an important adaptation strategy in regions that are vulnerable to climate variability and climate change.

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