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RESEARCH ARTICLE

Refinement of techniques for long-term storage of pollen from different coconut varieties in coastal Andhra Pradesh

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Abstract

The present investigation entitled "Refinement of techniques for long-term storage of pollen from different coconut varieties in coastal Andhra Pradesh" was conducted from 2021 to 2022 (November to June) at the College of Horticulture, Venkataramannagudem, West Godavari District, Andhra Pradesh. This study investigated the effects of different storage methods [room temperature, refrigerator (4 °C), freezer (-20 °C), ultra-low Freezer (-60 °C) and liquid nitrogen (-196 °C)] on the pollen of different coconut varieties, *viz.,* (ECT), (PHOT), (GBGD) and (COD) at 15 days intervals for up to 180 days of storage. The experiment was laid out in a Factorial Completely Randomized Design (FCRD) and mean separation was performed using Duncan's Multiple Range Test (DMRT) VIA SPSS 16.2. Pollen stored at room temperature and in a refrigerator is not viable after 180 days of storage. In contrast, the highest pollen viability %, germination percentage and pollen tube length were noted in liquid nitrogen stored pollen from the ECT (40.55±1.77 %, 39.96±1.32 % and 118.21±5.15µm), followed by the PHOT (38.98±1.70 %, 34.28±1.07% and 112.84±4.92µm) and GBGD (30.07±0.57 %, 29.87±1.21 % and 107.65±4.69µm) and the lowest values were observed in COD (28.35±0.55 %, 26.20±0.85 % and 102.05±4.45µm) pollen preserved in a freezer (-20 °C). In conclusion, using the liquid nitrogen storage method for the preservation of coconut pollen has proven to be a highly effective method for promoting better viability over the long term.

Keywords: coconut (*Cocos nucifera* L.); cryopreservation; pollen viability; pollen storage; ultralow temperature

Introduction

The coconut palm (*Cocos nucifera* L., 2n=32) is a perennial, woody monocot that belongs to the family Arecaceae and the subfamily Cocoideae, which includes 27 genera and 600 species (1). It is often called 'Kalpa Vriksha,' meaning 'the tree that provides all necessities of life,' also known as 'The Tree of Wealth' or 'The Tree of Life' (2). This palm is primarily found in coastal areas between 20 °N and 20 °S, at altitudes ranging from sea level to 1000 m. It is cultivated in more than 86 countries, divided into eight distinct coastal or oceanic regions across four continents (3). Globally, it is grown in more than 93 countries, covering 12.5 M ha and producing 67.6 million nuts, with an average yield of 5,387 nuts per hectare (4). In India, it is grown on 2.17 million hectares, yielding 20,308 million nuts with an average productivity of 9,345 nuts per hectare from 2021-2022.

The coconut palm holds great socioeconomic value and serves as a source of livelihood for millions of people in coconut -growing regions around the world (5). In coconut farming, hybrid varieties are created by crossbreeding tall and dwarf types using artificial pollination. Collecting pollen from the inflorescences of selected male parent lines is essential during hybridization, making the extraction technique and pollen quality critical factors in determining the success rate. The Influence of season on pollen production from individual male flowers and inflorescences. Pollen production from flowers is significantly greater in the summer and winter than in the rainy season (6). The collection and preservation of pollen is difficult even when pollen is highly available. The pollen stored at low temperatures and in a fresh state was used in the hybridization programme to achieve better success (7).

Enhancing the year-round availability of high-quality coconut pollen by implementing low-temperature storage techniques facilitates a continuous supply for breeding and the production of superior planting materials. This addresses the increasing demand for high-quality coconut resources among growers. Under natural conditions, coconut pollen viability diminishes within 48 hr. Hence, standardized storage methods to prolong viability are crucial (5). As a result, this study evaluated different low-temperature storage conditions for coconut pollen, aiming to increase its viability and enhance the production of high-quality seedling materials.

Materials and methods

Pollen collection from staminate flowers

The conventional method of collecting fresh pollen involves isolation of staminate flowers from the inflorescence of the respective palms. Staminate flowers (100 g) were weighed and kept on paper for crushing, which was accomplished by rolling

a bottle or a rolling pin. Crushed staminate flowers were placed in a sieving apparatus consisting of two sieves with mesh sizes of 500 and 212 microns, gently shaken to extract and sift the pollen from its floral parts and collected in a bottom pan. The obtained pollen was expressed in grams per 100 g of fresh staminate flowers (Fig. 1).

Treatments and storage conditions

The experiment was conducted using a factorial completely randomized design (FCRD) with two factors, resulting in twenty treatment combinations. Pollen samples were collected from each palm, wrapped in aluminium foil (0.02 g per sample) and placed into 2 mL cryovials. These cryovials were subjected to various low-temperature storage conditions: T_1:room temperature, T_2: refrigerator (4 °C), T_3: freezer (-20 °C), T_4: ultra-freezer (-60 °C) and T_5: liquid nitrogen (-196 °C) (Fig. 1). The pollen was stored for up to 180 days, with samples extracted every 15 days for analysis of pollen viability, germination and pollen tube length.

Pollen viability

To assess the viability of pollen grains through a staining method, approximately 0.02 g of pollen was placed on a slide and 1% acetic carmine was added. The slides were gently mixed to ensure the even distribution of the stain. The mixture was then placed on an 80 mm Petri dish (Labomax, Inc.) and incubated in a biological incubator for 25-30 min at 35±1 °C (8). The slides were examined under a 10x microscope (MAGCAM-DC3 model, fluorescence microscope with MagVision software) connected to a digital camera (MAGCAM-DC3, Magnus Analytics, New Delhi, India). Pollen viability was determined by counting viable and nonviable grains in each quadrant. Pollen grains stained red and had intact walls, indicating active enzymatic activity, were considered viable, whereas those with ruptured walls were classified as nonviable.

Pollen germination

To evaluate pollen germination, approximately 0.02 g of pollen was placed on Petri dishes containing 2 mL of culture media (9), which consisted of 0.2 g/L $MgSO_4.7H_2O$, 0.3 g/L $Ca(NO_3)_2.4H_2O$, 0.1 g/L KNO_3, 0.1g/L H_3BO_3 and 20 g/L sucrose. Pollen grains were transferred to slides with cotton and then positioned on Petri dishes lined with moist filter paper to maintain humidity. The Petri dishes were sealed and incubated for 1 hr or 30 min. When the incubation time was complete, the slides were removed from the Petri dish for to measure germination and it is done by using a microscope (model MAGCAM-DC3, fluorescence microscope with MagVision software) at 10x magnification and a digital camera (model MAGCAM-DC3, Magnus Analytics, New Delhi, India) were used.

Statistical analysis

The data from the study were subjected to analysis in a Factorial Completely Randomized Design (FCRD) by DMRT via the SPSS 16.2 software.

Results and Discussions

Statistically, highly significant differences were obtained among the storage conditions in which pollen was preserved, as well as duration, cultivars/varieties, for the interaction between storage conditions and cultivars. The current study aims to provide details on the best pollen storage conditions that might be employed to boost the potential for fertilization of selected coconut varieties. In coconut varieties, temperature affects the viability of pollen held for a long period. The results revealed significant variations in viability and germination, which were influenced by the coconut variety, storage conditions and the method employed for testing viability and germination. This study confirmed significant interactions between varieties and storage conditions; among the various storage conditions, pollen stored in liquid nitrogen (-196 °C) had the highest viability, germination percentage and pollen tube length after 180 days.

Pollen viability (%)

Since gametic selection affects the pollen of diverse populations during storage, it is crucial to establish storage conditions that minimize or eliminate differential selection (10). This can be achieved by storing the pollen at low temperatures, where cellular activities are halted (11). In the present study, notable differences were observed in the pollen viability of various coconut varieties subjected to different low-temperature storage conditions (Table 1). A rapid and permanent decline in pollen viability was evident after 15 days at room temperature. Conversely, pollen stored at low temperatures (4 °C, -20 °C, -60 °C and -196 °C) maintained viability until the 180th day. The longevity of pollen is largely determined by its genetic composition and is influenced by environmental factors (12). Pollen viability across plant species ranges from a few minutes to several years, depending on their taxonomic classification (13) and storage conditions. Research has demonstrated that low-temperature storage under controlled humidity can significantly increase pollen longevity in various horticultural and field crops (14). The percent viability obtained was higher in coconut pollen stored at -196 °C (34.49±6.17 %), followed by that stored at ultralow temperature (-60 °C) (31.00±6.73 %) and freezer (-20 °C) (22.89±5.09 %) (Table 1) after 180 days of pollen storage. On the other hand, significant varietal differences were noted, with East Coast Tall (ECT) showing the highest viability (21.30±19.91 %), followed by the Philippine Ordinary Tall (PHOT) (20.19±19.12 %).

The interaction effects were significantly pronounced, with East Coast Tall and Philippines Ordinary Tall demonstrating the highest viability (40.55±1.77 % and 38.98±1.71 %, respectively) after 180 days in liquid nitrogen. Similarly, some tall and dwarf coconut varieties maintained relatively high pollen viability at low temperatures and in liquid nitrogen for up to 60 days (15). Additionally, date palm pollen showed extended viability at -20 °C, exceeding that at 4 °C (16). These results align with the present study, where lower storage temperatures (-196 °C, -60 °C and -20 °C) proved more effective in preserving coconut pollen viability than storage at 4 °C. Subzero temperatures have also been shown to better preserve pollen viability in various other crops, including Cherimoya (9), Mango (17), Hazel (18) and Date Palm (19).

Pollen germination (%)

Pollen germination is expected to remain stable during prolonged storage at low temperatures. However, in the present study, significant differences were noticed in the pollen

Fig. 1. Procedure for cryopreservation of coconut pollen. **a**) Collection of spikelets in different coconut varieties; **b**) collection of pollen; **c**) pollen transfer for cryovial; **d**) pollen storage in liquid nitrogen (-196°C); **e**) viability test; **f**) observation of viable pollen in fluorescence microscope.

Table 1. Effects of different storage methods on pollen viability in different coconut varieties after 15 and 180 days of storage.

Storage methods	Per cent viability after 15 days					Per cent viability after 180 days				
	ECT	PHOT	GBGD	COD	Mean	ECT	PHOT	GBGD	COD	Mean
Room temperature	62.51±0.95^{g} (52.22)	55.76±1.15^{h} (48.28)	48.95±2.13^{i} (44.37)	45.96±2.01^{i} (42.66)	53.29±7.39^{e} (46.88)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{d} (0.00)
Refrigerator (4°C)	72.03±1.20^{d} (58.05)	67.57±0.60^{ef} (55.26)	50.26±2.19^{i} (45.12)	48.16±2.10^{i} (43.93)	59.51±12.05^{d} (50.59)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{f} (0.00)	0.00±0.00^{d} (0.00)
Freezer (-20°C)	77.63±0.90^{c} (61.74)	69.45±0.71^{def} (56.42)	65.48±2.85^{fg} (54.00)	62.45±2.72^{g} (52.19)	68.75±6.57^{c} (56.09)	28.78±0.94^{c} (32.42)	25.48±25.48^{d} (30.30)	19.13±0.24^{e} (25.92)	18.18±0.72^{e} (25.22)	22.89±5.09^{c} (28.47)
Ultra-low Freezer (-60°C)	81.61±1.00^{bc} (64.58)	77.73±1.05^{c} (61.75)	70.12±3.06^{de} (56.86)	68.54±2.99^{def} (55.87)	74.50±6.21^{b} (59.78)	37.15±1.62^{b} (37.53)	36.49±36.49^{b} (37.14)	25.55±0.12^{d} (30.34)	24.18±2.07^{d} (29.92)	31.00±6.73^{b} (33.17)
Liquid nitrogen (-196°C)	92.64±1.61^{a} (74.04)	84.46±0.71^{b} (66.82)	78.88±3.44^{c} (62.66)	72.02±3.14^{d} (58.06)	81.95±8.66^{a} (65.38)	40.55±1.77^{a} (39.53)	38.98±1.70^{a} (38.16)	30.07±0.57^{c} (33.24)	28.35±0.55^{c} (32.15)	34.49±6.17^{a} (35.88)
Mean	77.25±11.12^{a}	70.99±10.87^{b}	62.73±12.93^{c}	59.43±11.82^{d}		21.30±19.91^{a}	20.19±19.12^{b}	14.94±14.19^{c}	14.26±13.53^{d}	
Factors					LSD					LSD
Storage methods					0.94					0.46
Varieties					1.05					0.51
SM×V					2.16					1.03

ECT-East Coast Tall, **PHOT**-Philippines Ordinary Tall, **GBGD**-Ganga Bondam Green Dwarf, **COD**-Chowghat Orange Dwarf, **SM**-Storage Methods and **V**-Varieties. Mean values of parameters followed by the same letter are not significantly different according to the DMRT ($P \leq 0.05$).

germination of different coconut varieties when subjected to low temperature storage (Table 2). As the storage period increased, a slight decrease in the germination percentage of pollen from various coconut varieties was observed across most low-temperature treatments. Conversely, a significant decline in germination was noted for pollen stored at room temperature after just 15 days, which became permanent. After 180 days of storage, the highest germination percentage was recorded for coconut pollen preserved at -196 ºC (32.58±5.93 %), followed by coconut pollen stored at ultra-low temperatures at -60 ºC (27.49±6.32 %) and freezer storage at -20 ºC (21.29±6.32 %) (Table 2). On the other hand, the significant varietal differences were noted, with East Coast Tall (ECT) showing the highest germination percentage (20.87±19.47 %), followed by Philippine Ordinary Tall (PHOT) (17.26±16.20 %). The interaction effects were highly significant, with East Coast Tall and Philippines Ordinary Tall showing the highest germination percent (39.96±1.32 % and 34.28±1.07 %, respectively) in liquid nitrogen after 180 days of storage. The current study's successful cryopreservation of coconut pollen corresponds with previous research (20). Pollen from West Coast Tall (WCT) and Chowghat Orange Dwarf (COD) varieties retained viability levels of 24-32 % and 30-31 %, respectively, after cryopreservation (21). Similar successes in cryogenic pollen storage have been reported for other palms, including date palm (19, 22, 23) and oil palm (24). Likewise, the cryopreservation of tricellular pollen has been effectively achieved in coconut (25), cherimoya (9) and onion (26). The differences in cryo-storage results among coconut varieties can likely be attributed to their distinct capacities to endure low-temperature stress.

Pollen tube length (µm)

The pollen tube length of different coconut varieties was significantly influenced by storage conditions, as assessed through *in vitro* germination. Based on the findings (Table 3, Fig. 2 and 3), pollen stored at -196 ºC exhibited the most extended average pollen tube length (110.19±6.93µm), followed by storage at -60 ºC (101.80±5.38µm) and -20 ºC (93.86±4.75µm) after 180 days. Among the varieties, East Coast Tall (ECT) recorded the highest pollen tube length (65.47±60.12µm), followed by Philippine Ordinary Tall (PHOT) (61.98±56.91µm). A significant interaction effect was also observed, with East Coast Tall and Philippine Ordinary Tall showing maximum pollen tube lengths of 118.21±5.15µm and 112.84±4.92µm, respectively, when stored in liquid nitrogen for 180 days. The cryopreserved pollen tube lengths of the study's Tall and Dwarf coconut varieties showed discrepancies. The variation in pollen tube length made the genotypic variety of several Prunus species obvious (27). Diminished pollen tube growth was detected in *Lilium longiflorum* pollen stored at low temperatures, primarily due to the rapid degradation of preformed proteins and mRNAs during the cold storage (28). Similar observations have been made in coconut (19), peach (29), plum, almond and apricot (30), in cotton and almond, apricot and sweet cherry genotypes (31). Pollen from various coconut varieties, including ECT, PHOT, COD and GBGD, can be effectively preserved for up to 180 days at temperatures of -196 ºC (in liquid nitrogen), -60 ºC (in an ultra-low freezer) and -20 ºC (in a freezer), with minimal reduction in viability and germination potential.

Table 2. Effects of different storage methods on pollen germination in different coconut varieties after 15 and 180 days of storage.

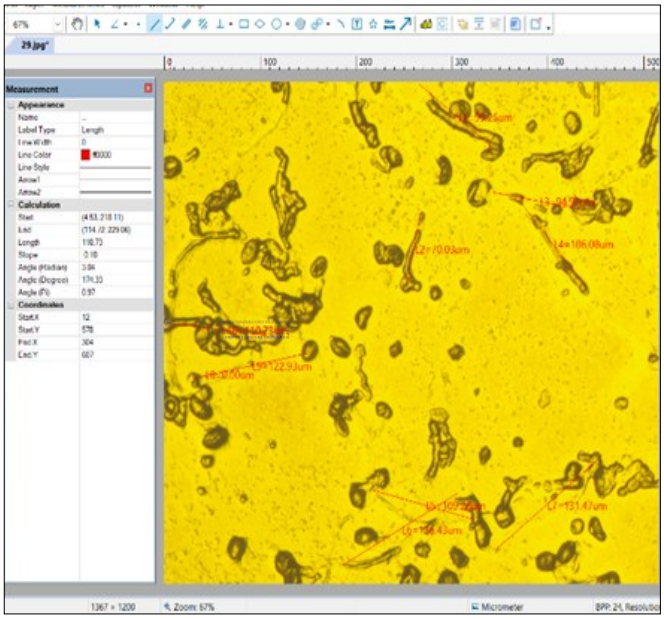
Storage methods	Germination percentage after 15 days					Germination percentage after 180 days				
	ECT	PHOT	GBGD	COD	Mean	ECT	PHOT	GBGD	COD	Mean
Room temperature	32.22±0.31^{g} (34.56)	29.56±1.31^{h} (32.91)	21.70±0.42^{j} (27.55)	17.10±0.85^{k} (24.41)	25.14±6.98^{e} (29.91)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{e} (0.00)
Refrigerator (4°C)	37.70±0.59^{e} (37.86)	31.72±0.60^{g} (34.26)	24.15±0.38^{i} (29.42)	18.86±0.58^{k} (25.72)	28.11±8.29^{d} (31.81)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{j} (0.00)	0.00±0.00^{d} (0.00)
Freezer (-20°C)	41.46±1.08^{d} (40.06)	34.44±0.88^{f} (35.91)	28.60±1.27^{h} (32.31)	23.34±1.00^{i} (28.87)	31.96±7.79^{c} (34.29)	28.69±0.52^{e} (32.37)	23.66±0.70^{g} (29.09)	18.86±0.80^{h} (25.72)	13.97±0.65^{i} (21.90)	21.29±6.32^{c} (27.28)
Ultra-low Freezer (-60°C)	49.31±0.77^{c} (44.58)	42.71±1.14^{d} (40.79)	34.31±0.80^{f} (35.83)	32.39±0.55^{g} (34.67)	39.68±7.83^{b} (38.97)	35.71±0.51^{b} (36.60)	28.38±0.58^{e} (32.17)	26.87±0.73^{f} (31.20)	19.00±0.44^{h} (25.80)	27.49±6.85^{b} (31.47)
Liquid nitrogen (-196°C)	59.75±0.99^{a} (50.60)	55.98±0.94^{b} (48.41)	42.27±1.40^{d} (40.53)	38.92±1.09^{e} (38.58)	49.23±10.18^{a} (30.45)	39.96±1.32^{a} (39.18)	34.28±1.07^{c} (35.82)	29.87±1.21^{d} (33.11)	26.20±0.85^{f} (30.77)	32.58±5.93^{a} (34.72)
Mean	44.09±10.74^{a} (41.53)	38.88±10.78^{b} (38.46)	30.20±8.27^{c} (33.17)	26.12±9.29^{d} (30.45)		20.87±19.47^{a} (21.64)	17.26±16.20^{b} (19.41)	15.12±14.38^{c} (18.01)	11.83±11.64^{d} (15.70)	
Factors			LSD					LSD		
Storage methods			0.41					0.30		
Varieties			0.46					0.34		
SM×V			0.92					0.68		

ECT-East Coast Tall, **PHOT**-Philippines Ordinary Tall, **GBGD**-Ganga Bondam Green Dwarf, **COD**-Chowghat Orange Dwarf, **SM-**Storage Methods and **V**-Varieties. Mean values of parameters followed by the same letter are not significantly different according to the DMRT (P ≤ 0.05).

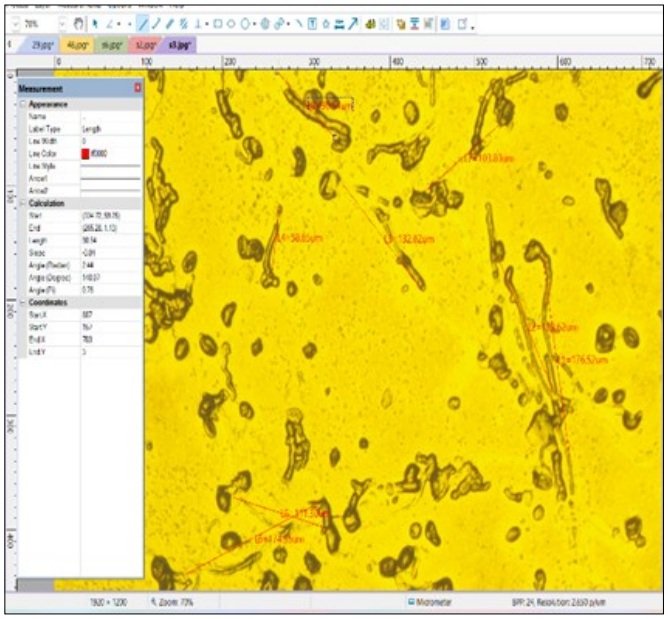
Table 3. Effects of different storage methods on pollen tube length in different coconut varieties after 15 and 180 days of storage.

Storage methods	Pollen tube length (µm) after 15 days					Pollen tube length (µm) after 180 days				
	ECT	PHOT	GBGD	COD	Mean	ECT	PHOT	GBGD	COD	Mean
Room temperature	145.48±6.34^{hij}	142.01±6.19^{hij}	135.06±5.89^{j}	130.40±5.68^{j}	138.24±6.79^{e}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{d}
Refrigerator (4°C)	154.73±6.74^{gh}	151.47±6.60^{ghi}	141.74±6.18^{hij}	137.95±6.02^{i}	146.48±7.92^{d}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.00^{d}
Freezer (-20°C)	182.54±7.96^{ef}	180.25±7.86^{ef}	160.91±7.02^{g}	158.96±6.93^{g}	170.67±12.45^{c}	99.65±4.34^{d}	95.74±4.17^{de}	90.85±3.96^{ef}	89.21±3.89^{f}	93.86±4.75^{c}
Ultra-low Freezer (-60°C)	193.46±8.43^{de}	190.54±8.31^{def}	180.35±7.86^{ef}	177.54±7.74^{f}	185.47±7.72^{b}	109.48±4.77^{b}	101.31±4.42^{d}	99.15±4.32^{d}	97.25±4.24^{d}	101.80±5.38^{b}
Liquid nitrogen (-196°C)	230.15±10.03^{a}	220.51±9.62^{ab}	212.31±9.26^{bc}	200.66±8.74^{cd}	215.91±12.51^{a}	118.21±5.15^{a}	112.84±4.92^{ab}	107.65±4.69^{bc}	102.05±4.45^{cd}	110.19±6.93^{a}
Mean	181.27±33.63^{a}	176.96±31.48^{b}	166.07±31.31^{c}	161.10±28.80^{d}		65.47±60.12^{a}	61.98±56.91^{b}	59.53±54.67^{c}	57.70±52.87^{d}	
Factors			LSD					LSD		
Storage methods			5.61					2.55		
Varieties			6.27					2.86		
SM×V			11.18					5.71		

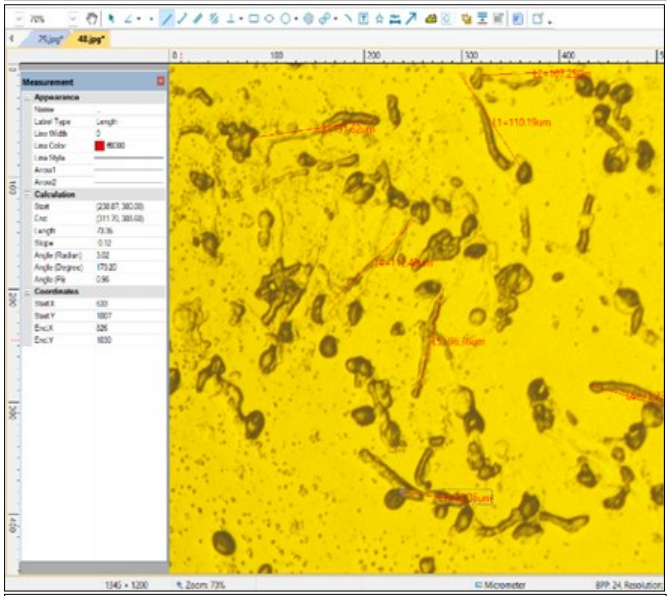
ECT-East Coast Tall, **PHOT**-Philippines Ordinary Tall, **GBGD**-Ganga Bondam Green Dwarf, **COD**-Chowghat Orange Dwarf, **SM**-Storage Methods and **V**-Varieties. Mean values of parameters followed by the same letter are not significantly different according to the DMRT (P ≤ 0.05).



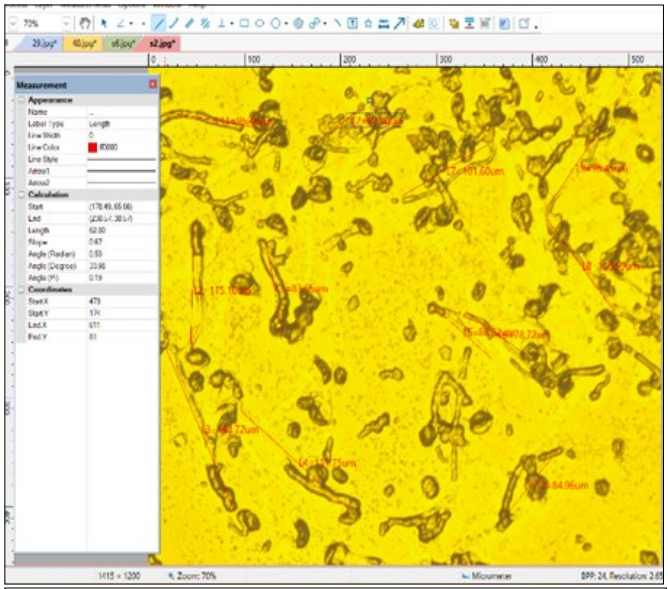
Room temperature



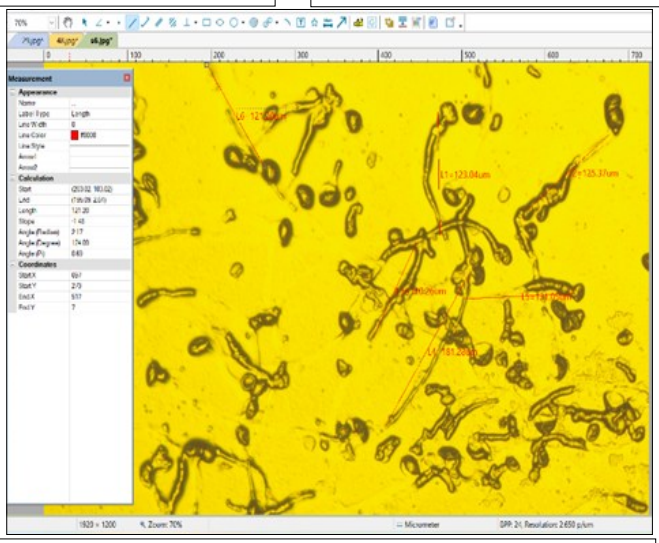
Refrigerator (4°C)



Freezer (-20°C)

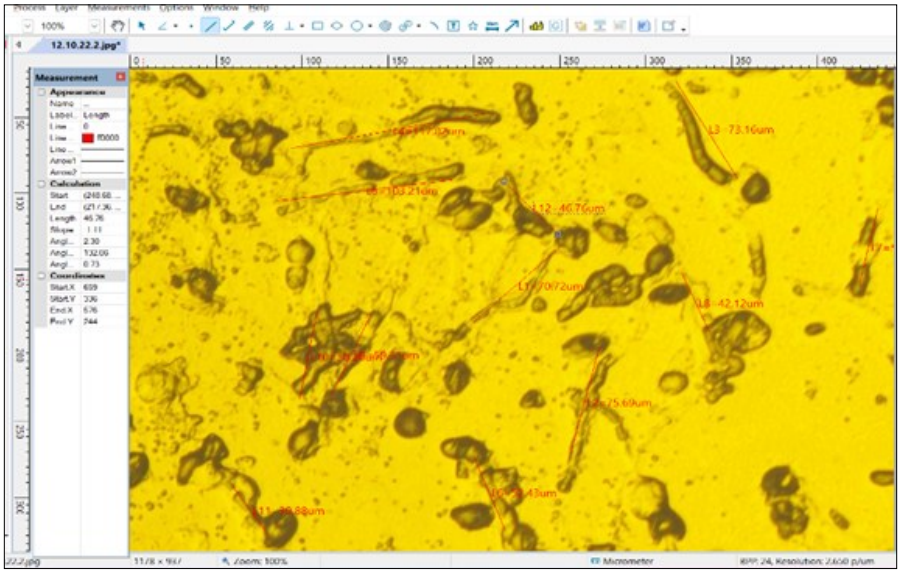


Ultra-low freezer (-60°C)

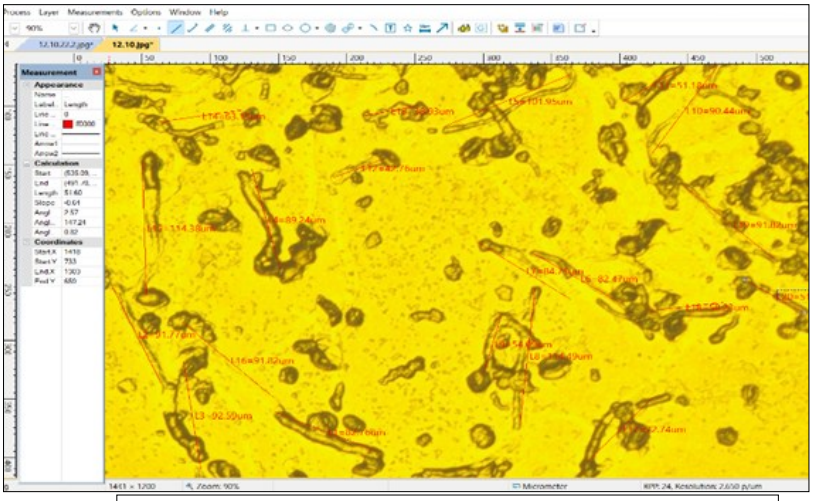


Liquid nitrogen (-196°C)

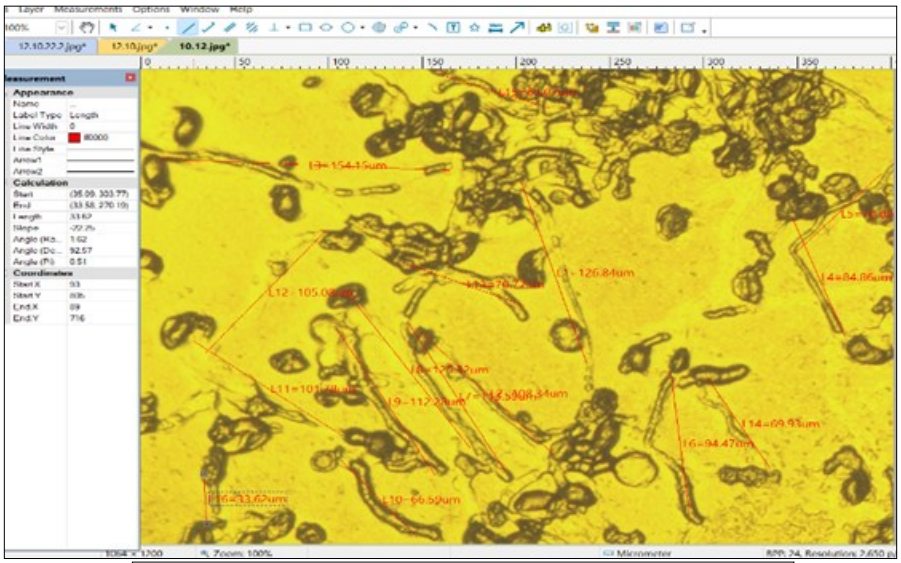
Fig. 2. Pollen tube length (µm) as affected by different storage conditions (after 15 days of storage). **1**).Room temperature, **2**).Refrigerator (4°C), **3**).Freezer (-20°C), **4**).Ultra-low freezer (-60°C) and **5**).Liquid nitrogen (-196°C).



Freezer (-20°C)



Ultra-low freezer (-60°C)



Liquid nitrogen (-196°C)

Fig. 3. Pollen tube length (µm) as affected by different storage conditions (after 180 days of storage). **1**).Freezer (-20^{0}C), **2**).Ultra-low freezer (-60^{0}C) and **3**).Liquid nitrogen (-196^{0}C).

Conclusion

Pollen collection and preservation are vital for hybridization initiatives in coconut breeding. Developing an efficient short-term preservation method ensures the availability of coconut pollen for breeding throughout the year. This study involved collecting pollen from various coconut varieties through a standardized extraction method and examining its preservation under four low-temperature conditions: 4 °C, -20 °C, -60 °C and -196 °C, along with room temperature, over 180 days. The findings revealed that after 180 days, pollen stored at room temperature and in a refrigerator was no longer viable. In contrast, pollen stored in liquid nitrogen (-196 °C) from East Coast Tall varieties maintained the highest viability. These results suggest that coconut pollen can be effectively preserved for long-term use at -196 °C, supporting breeding programs. However, this study has certain limitations, including the assessment of pollen viability only up to 180 days, the focus on specific coconut varieties and the lack of evaluation of stored pollen's fertilization efficiency under field conditions. Future studies should explore the long-term viability of pollen beyond 180 days, assess its fertilization potential in hybridization programs and optimize storage techniques for a broader range of coconut genotypes.

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Authors' contributions

SP performed Conceptualization, Methodology and Writing Original draft preparation. KM, NNL, AV, PM and SV performed Supervision, reviewing and editing.

Compliance with ethical standards

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Ethical issues: None

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