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Original Research Paper

Exploring pollen cryopreservation, germination, and morphometry of mango (*Mangifera indica* L.) Appemidi genotypes

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ABSTRACT

Pollen cryopreservation is a vital technique for maintaining genetic diversity in highly heterozygous crops like mango, ensuring the long-term sustainability of crop improvement programs. The present study examined the effects of cryopreservation of pollen from mango Appemidi genotypes, to enhance and protect the mango gene pool. Dehisced and near-dehisced anthers were collected during the optimal time frame of 10:30–11:30 am. *In vitro* pollen germination assays revealed significant variability among genotypes, with germination ranging from 10.84% to 85.46%. Notably, the genotype Arenuru exhibited the highest pollen germination rate and greatest pollen tube elongation after one month of cryopreservation. Further, scanning electron microscopy, revealed significant structural variations among the accessions. Overall, this study highlights the efficacy of pollen cryopreservation as a viable strategy for conserving genetic diversity and its potential contribution to future mango breeding and improvement programs.

Keywords: Appemidi, cryopreservation, pollen germination, genotype, diversity

INTRODUCTION

Genetic improvement in any plant breeding program hinges on the availability and conservation of plant genetic resources. Cryopreservation serves as a pivotal tool in preserving diversity *in vitro*. Within the realm of plant genetic conservation, pollen cryopreservation emerges as a complementary strategy, particularly applicable to preserving diversity within the genus *Mangifera*. Mango, a highly heterozygous and diverse crop, harbours its diversity across various regions, notably enriched in the Western Ghats regions of Karnataka, known for its unique aromatic pickle-type mangoes (Vasugi et al., 2012). These wild types are acknowledged as reservoirs of valuable genes crucial for adaptation to climate change and the emergence of new biotypes.

Appemidi a tender pickling mango, occupies a distinctive position in the diversity of pickling mango varieties in Karnataka. Its fragrance is so potent that adding a few tender mango fruits to an ordinary pickle can significantly alter its taste and aroma. The rich diversity of Appemidi is predominantly found in the Western Ghats regions of Karnataka, India (Dinesh et al., 2015).

Among the myriad conservation strategies for plant genetic resources, pollen cryopreservation of perennial trees holds significant importance due to its potential genetic traits, applicable in breeding programs. Cryopreserved pollen can bridge the temporal gap between male and female flowering periods, enhancing fruit setting in orchards. Furthermore, cryopreserved pollen serves as a germplasm resource, facilitating distribution and exchange among breeders. Cryopreservation stands out as the most efficient method for the long-term conservation of pollen grains, offering indefinite longevity and reportedly causing no genetic damage (Towill, 1985; Ganeshan et al. 2008; Rajashekaran & Ganeshan, 2019).

This study focuses on optimizing pollen germination media for surveyed Appemidi types and cryopreservation to safeguard the nuclear genetic pool of mango. The primary objective is to conserve these unique mango types *in vitro* and *ex situ*, specific to the Western Ghats, Karnataka. However, the lack of awareness among the local populace poses a threat to this unique diversity heritage, resulting in damage due to human activities. The indiscriminate cutting of branches for fruit harvesting, particularly of Appemidi, which holds significant market value, exacerbates the

situation. A single fruit can fetch prices ranging from 5 to 10 INR. Thus, a survey was conducted, and unique scion types as well as pollen were collected for conservation purposes. Additionally, apart from *in vitro* conservation, pollen germination and pollen morphometry using scanning electron microscopy were studied and documented.

MATERIALS AND METHODS

The study was conducted at Cryopreservation Laboratory, of ICAR-Indian Institute of Horticultural Research, Bengaluru, India, spanning from 2016 to 2018. The experimental material was gathered through systematic surveys conducted in the Western Ghats regions of Chikkamagluru, Karnataka, India. Thirteen distinct collections of Appemidi were obtained from thirteen different locations, with both pollen and scions collected for *ex situ* and *in situ* conservation. Anthesis and anther dehiscence were documented in the surveyed Appemidi collections.

Mature male and hermaphrodite flowers in full bloom were harvested from healthy panicles between 10 and 11 am. These flowers were placed individually into petri dishes and exposed to direct sunlight for approximately 10-15 minutes to expedite anther dehiscence. During the survey, fully bloomed male flowers were collected, packed in butter paper bags, and stored in ice pack boxes to maintain viability. After 24 hours, these flowers were transferred to petri plates and exposed to direct sunlight for 15-20 minutes to facilitate anther dehiscence and moisture removal.

Freshly dehisced cryopreserved pollen was subjected to germination trials using various media combinations employing hanging drop and sitting drop techniques. The nutrient media comprised 1 mg boric acid, 1.5 g sucrose, 3 mg calcium nitrite ($CaNo_3$), and 2 mg magnesium sulphate ($MgSO_4$) per 10 mL of distilled water, prepared a day prior and refrigerated for further use. Sucrose concentrations of 5% and 10%, along with water as a control, were tested. Each media combination was replicated thrice, and germination was assessed after 24 hours of incubation under 10X magnification. Pollen grains with tube lengths exceeding their diameters were considered germinated and viable. Pollen germination percentage was calculated by dividing the number of germinated pollens per field of view by the total number of pollens per field of view and expressed as a percentage. The experimental design was a completely randomized design. Germination data were arc sine transformed ($\sqrt{x}/100$) for statistical analysis, with means compared using analysis of variance and the Scott-Knott test ($p \leq 0.01$) through SAS Institute software.

Dehisced pollen was pooled and transferred into gelatine capsules, then packed into airtight aluminium foil pouches with proper labelling before immersion in liquid nitrogen at -196°C within a cryobank canister for long-term conservation.

Freshly dehisced pollen grains were affixed onto double-sided copper tape on polished aluminium stubs and subjected to vacuum evaporation in an SEM microscope (HITACHI-TM3030Plus). Pollen grains were observed and photographed at magnifications ranging from 1000X to 5000X. For each genotype, 10-15 pollen grains were analyzed to determine size and morphological traits, including equatorial diameter (E), polar axis (P), P/E ratio, exine sculpture ornamentation, length of colpi (C), and colpi percentage (CP), utilizing the terminology of Erdtman (1971) and Esme et al. (1986).

RESULTS AND DISCUSSION

Anthesis & anther dehiscence

The study revealed that anthesis typically commenced between 6:30 to 8:30 am, with the peak period for pollen collection occurring around 10:30 to 11:30 am when anthers were about to dehisce (Table 1). Based on the cultivar type and weather parameters, mainly temperature plays a major role in accelerating the activity of anthesis and anther dehiscence (Pimentel et al., 1984; Indu Bala, 1990). Our observations were also in line with Geetha et al. (2016), which indicated

Table 1 : Events of anthesis

Event	Time interval	Observations
Commencement of anthesis	6.30 am - 8.30 am	Initial flower buds opening
Anther dehiscence	8.00 am - 12.00 pm	Soon after anthesis, the process of anther dehiscence will start
Peak period of anther dehiscence	10.30 am - 11.30 am	Peak time of anther dehiscence

Table 2 : Pollen morphometry of surveyed Appemidi genotypes from Western Ghat regions of Chikkamagluru, India

Genotype	Pollen length (µm)	Equatorial diameter (µm)	Colpi length (µm)	Colpi (%)	P/E ratio	Mean size (mm)	Pollen shape
Arenuru-2	59.92	25.67	47.83	79.83	2.33	1.54	Perprolate
Bynduru	54.14	20.36	47.49	87.72	2.66	1.10	Perprolate
Chimankudige-3	47.80	22.42	41.65	87.14	2.13	1.07	Prolate
Kaman Mori-1	47.03	24.05	37.18	79.06	1.96	1.13	Prolate
Doobla	54.28	22.85	38.32	70.60	2.37	1.24	Perprolate
Elemadlu-1	52.11	19.59	39.79	76.36	2.66	1.02	Perprolate
Gonibeedu-1	50.60	23.13	40.87	80.77	2.19	1.17	Perprolate
Marebylu-2	54.32	22.48	45.68	84.10	2.42	1.22	Perprolate
Kudige	58.89	23.70	48.40	82.19	2.49	1.40	Perprolate
Heruru-1	45.61	24.67	36.43	79.88	1.85	1.13	Prolate
Kumrumane-2	49.62	24.56	35.30	71.13	2.02	1.22	Perprolate
Magodu	51.84	24.36	35.80	69.05	2.13	1.26	Perprolate
Soppinakadu-3	42.19	25.19	38.27	90.72	1.67	1.06	Prolate
Mean	50.65	23.14	40.47	80.02	2.21	1.17	
S.E. (m)±	1.40	0.50	1.30	1.80	0.08	0.08	
C.D. at 1%	0.38	0.19	0.48	1.27	0.03	0.03	
F-Test	*	*	*	*	*	*	
C.V.	0.47	0.52	0.75	1.02	0.85	1.42	

that maximum anthesis occurs in early morning hours coupled with high temperature (De Wet & Robbertse 1986). But the anther dehiscence was at its peak at 10.30 to 11.30 AM in the surveyed areas.

Optimization of media for *in vitro* pollen germination

Pollen germination media were optimized for cryopreserved Appemidi genotypes collected from 13 different locations varying in sucrose and boric acid concentrations. Table 2 presents the results of *in vitro* pollen germination for 13 different genotypes of Appemidi mangoes. The data reveal considerable variation in pollen germination rates among the different genotypes, ranging from 10.84% to 85.46%. Similarly, pollen tube lengths vary significantly, with values ranging from 35.25 µm to 115.65 µm (Table 2). The length and width of the pollen heads also exhibit variability across the genotypes, reflecting diverse morphological characteristics.

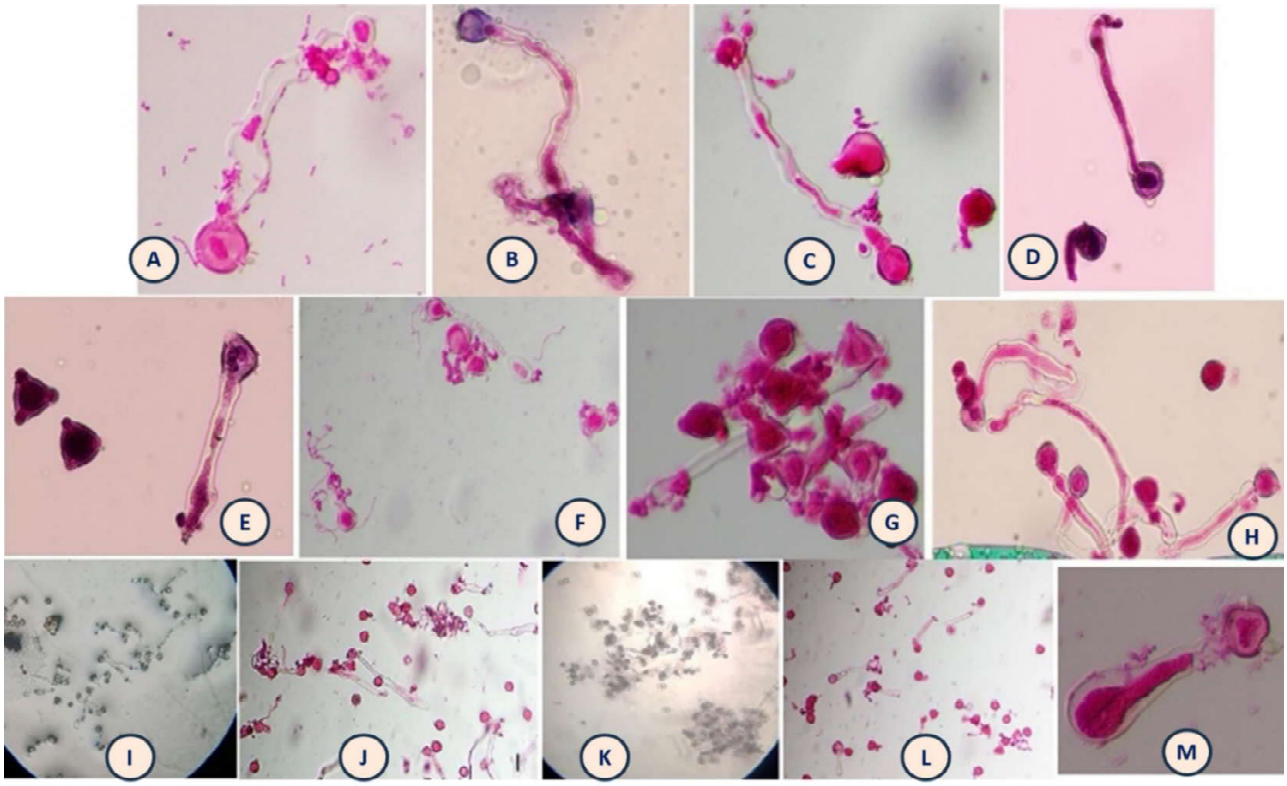
Establishing an appropriate *in vitro* germination protocol was crucial before attempting pollen cryopreservation. Results showed higher pollen germination rates in a medium containing 15% sucrose, 100 ppm boric acid, and 300 ppm $CaNO_3$. Optimal germination was observed after 24 hours of incubation at room temperature. These findings provide valuable insights for further studies on mango breeding and conservation efforts.

Effect of cryopreservation on pollen germination

Following collection, pollen was exposed to sunlight for 15-20 minutes, well-packed, and cryopreserved. After one-month, cryopreserved pollen from the 13 Appemidi genotypes exhibited positive responses during *in vitro* germination assays. Most of the surveyed and cryopreserved pollen displayed robust germination under *in vitro* conditions after 24 hours of incubation. Notably, varied germination profiles were observed among the 13 genotypes, with genotype Arenuru exhibiting the highest germination percentage (85.46%) and Chimankudige the lowest (10.84%) (Table 2). Cryopreserved pollen was exclusively used for *in vitro* germination assessments to evaluate the viability of surveyed pollen grains. Additionally, pollen tube length, head length, and head width were measured. The maximum pollen tube growth was observed in Arenuru (115.65 µm), with respective head dimensions of 33.51 µm and 29.61 µm (Table 2 & Fig. 2).

Table 3 : *In vitro* pollen germination of different Appemidi genotypes after a month of cryopreservation

Genotype	Pollen germination (%)	Pollen tube length (µm)	Pollen head length (µm)	Pollen head width (µm)
Bynduru	26.94	58.95	26.17	28.5
Kamanmori	13.25	35.25	25.11	26.2
Chimankudige	10.84	40.5	31.1	30.05
Arenuru	85.46	115.65	33.51	29.11
Elemadlu	58.21	65.55	10.65	12.53
Kudige	84.58	74.1	30.5	29.15
Magodu	83.38	70.55	29.85	28
Heruru	36.63	100.74	31.55	20.11
Kumrumane	80.1	106.38	26.57	28.05
Doobla	40.56	75.83	31.54	28.97
Soppinakadu	20.84	110.46	9.46	10.52
Gonibeedu	50.65	69.01	9.65	12.55
Marebylu	52.22	39.21	10.16	10.2
Mean	49.51	74.01	23.52	22.611
SEm(±)	7.63	7.60	2.69	2.26
F Test	*	*	*	*



A: Bynduru, B: Kamanmori, C: Chimankudige, D: Soppinakadu, E: Doobla, F: Magodu, G: Gonibeedu, H: Heruru, I: Kumrumane, J: Arenuru, K: Elemadlu, L: Kudige, M: Marebylu

Fig. 1 : Variability in pollen head length, width and tail length after pollen germination of surveyed Appemidi genotypes

Data presented in Table 1 summarizes the morphometric characteristics of pollen grains from various Appemidi genotypes collected in the Western Ghats regions of Chikkamagluru. The data revealed significant diversity among the surveyed genotypes in terms of pollen size, shape, and colpi characteristics. For instance, pollen length varies from 42.19 µm to 59.92 µm, while equatorial diameter ranges from 19.59 µm to 25.67 µm. The colpi length varies from 35.30 µm to 48.60 µm, with colpi percentages ranging from 69.05 to 98.34. The genotype Arenuru-2 displayed the maximum pollen length (59.92 µm), comparable to Kudige (58.89 µm), while, Soppinakadu-3 exhibited the minimum (42.19 µm). Equatorial width showed significant differences, with Marebilu-3 recording the maximum (27.70 µm) and Elemadlu-1 the minimum (19.59 µm). Genotypes also varied significantly in colpi length and percentage, with Heruru-2 exhibiting the longest colpi (48.60 µm) and highest colpi percentage (98.34%), and Doobla the lowest (70.60%). P/E ratio varied among genotypes, with Bynduru and Elemadlu-1 displaying the highest ratios (2.66), indicative of perprolate pollen shape, while Soppinakadu-3 and Marebylu-3 had the lowest ratios (1.67 and 1.74, respectively), suggesting prolate pollen shape (Table 1). Maximum pollen size was recorded in Arenuru-2 (1.54 mm), while Kumrumane-1 exhibited the minimum. Exine ornamentation appeared thick, striate to reticulated, with some genotypes displaying large perforations on the outer surface (Fig. 1). Overall, these measurements provide valuable insights into the genetic diversity and morphological variability of Appemidi pollen grains, which are crucial for taxonomic classification and further genetic studies.

The success of pollen cryopreservation hinges on precise flower collection timing and pollen maturity, which varied among the surveyed genotypes. *In vitro* germination profiles were influenced by factors such as sucrose concentration in the culture media, composition, collection time, method, and incubation period. In this study, optimal germination was observed with 1.5 g sucrose and boric acid, indicating their significance in Appemidi mango *in vitro* germination. While fresh pollen germination *in vitro* was not attempted, the promising response post-cryopreservation underscores the viability of these unique types, facilitating conservation and further research without damage.

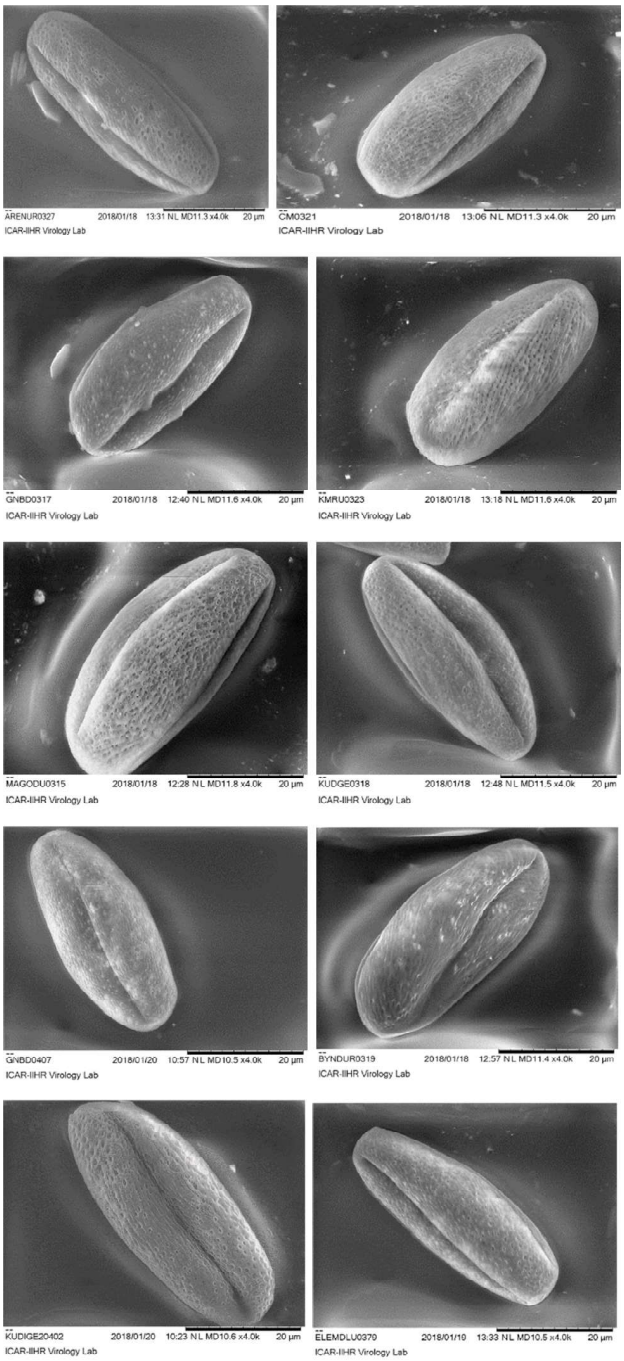


Fig. 1 : Scanning electron microscopic images of surveyed Appemidi pollen

Pollen tube length exceeding head diameter signifies high fertility and viability, a trait observed across all 13 unique genotypes. Cryopreservation demonstrated its potential for pollen conservation, with cryopreserved pollen displaying active germination profiles even after a month of ultra-low temperature storage. Theoretically, cryopreserved pollen can maintain viability indefinitely at -196°C, ensuring

prolonged availability of potent pollen. Pollen grains showed striate to reticulate type exine sculpture with perforations in the grooves (Fig. 2) and these results were confirmed by Singh (1954), Randhawa & Damodaran (1961), Bhattacharya et al. (2009), Bhattacharya et al. (2012). Pollen grains have several common features, including being prolate or perprolate; however, they differed in exine ornamentation (Yang et al., 2015). The major evolutionary trend of exine sculpture is postulated to be from reticulate through regulate to striate-reticulate or vice versa, in Alstroemeriacese reported by Aagesen & Sanso (2003). There is an existence of a general relationship between pollen morphology and pollen vectors as suggested by Wood house (1935), that the reticulate exine sculpture might represent the mode of pollination.

Cryopreservation is emerged as a pivotal tool for conserving the diverse heritage of mango without causing harm because at ultra-low temperature plant samples can be stored for longer time without any damage (Mortazavi et al., 2010) Scanning electron microscopy facilitated morphological characterization of pollen grains, providing valuable taxonomic information. Significant differences were noted among genotypes for colpi length and percentage, P/E ratio, and pollen shape, with variations in exine ornamentation. This study emphasizes the importance of conservation efforts to safeguard our rich botanical heritage and underscores cryopreservation as a valuable technique in this endeavour. Further research and conservation initiatives are warranted to ensure the preservation of endangered plant species like Appemidi mango.

CONCLUSION

This study successfully achieved pollen cryopreservation in 13 genotypes of Appemidi, each exhibiting varying *in vitro* germination profiles. The present study effectively conserved the unique aromatic pickle mango genotypes found in the Western Ghats regions of Karnataka, both *ex situ* and *in situ* environments. Additionally, the establishment of a pollen cryobank for Appemidi mango has been accomplished, serving as a vital gene source for breeders and facilitating further research endeavours.

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