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COCONUT (*COCOS NUCIFERA* L.) POLLEN CRYOPRESERVATION

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Abstract

BACKGROUND: Coconut genetic resources are threatened by pests and pathogens, natural hazards and human activities. Cryopreservation is the only method allowing the safe and cost-effective long-term conservation of recalcitrant seed species such as coconut. **OBJECTIVE:** The objective of this work was to test the effect of cryopreservation and of cryostorage duration on coconut pollen germination and fertility. **MATERIALS AND METHODS:** Pollen of two coconut varieties (West Coast Tall [WCT] and Chowghat Orange Dwarf [COD]) was collected in March-May over three successive years, desiccated to 7.5% moisture content (FW) and cryopreserved by direct immersion in liquid nitrogen. **RESULTS:** Germination and pollen tube length (PTL) of desiccated and cryopreserved pollen were not significantly different for both WCT and COD over the three harvest months of the three consecutive years of study. Pollen germination ranged from 24 to 32% in desiccated pollen whereas it was between 26 and 29% in cryopreserved COD pollen. In the case of WCT, germination ranged from 30 to 31% in desiccated pollen, while it was between 28 and 32% in cryopreserved pollen. PTL of cryopreserved pollen ranged between 224-390 μm and 226-396 μm for COD and WCT, respectively. Germination of COD pollen varied between 29.0 and 44.1% after 4 years and 1.0/1.5 years cryostorage, respectively. Germination of WCT pollen did not change significantly between 0 and 6 years cryostorage, being comprised between 32 (24 h) and 40 % (1.5 years). Germination and vigour of cryopreserved pollen were generally higher compared to that of pollen dried in oven and non-cryopreserved. Normal seed set was observed in COD and WCT palms using pollen cryostored for 6 months and 4 years. Cryopreserved pollen of five Tall and five Dwarf accessions displayed 24-31% and 25-49% germination, respectively. **CONCLUSION:** These results show that it is now possible to establish pollen cryobanks to contribute to coconut germplasm long-term conservation.

Keywords: coconut, pollen, cryopreservation, germination, vigour, fertility

INTRODUCTION

Coconut (*Cocos nucifera* L.), popularly known in India as "*Kalpa Vrisha*", provides mankind with all the necessities of life. Nevertheless the coconut industry is currently passing through a challenging phase because of the low productivity of ageing plantations and the occurrence of new pests and diseases. This induces a low and fluctuating income generation for farmers who are compelled to shift land use

for more profitable crops. Consequently, coconut genetic diversity decreases gradually, thus hampering its utilization for breeding purposes. As coconut seeds are recalcitrant, the traditional approach for *ex situ* conservation of coconut genetic diversity is field genebanks. Field genebanks allow ready availability of plant material for characterization, evaluation and use. However, genetic resources are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism (45). In addition, they are not in a condition that is readily conducive to germplasm exchange because of the great risks of disease transfer through the exchange of vegetative material. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency. Hence there is an urgent need to develop complementary methods to ensure the conservation of the genetic diversity present in the existing main coconut varieties/cultivars threatened with genetic erosion or loss, or with special traits/genetic markers.

Cryopreservation is the only method allowing the safe and cost-effective long-term conservation of recalcitrant seed species such as coconut (14). In the case of coconut, most cryopreservation studies have used zygotic embryos as explants (15). Pollen is another valuable source of diverse alleles within a gene pool. Pollen cryopreservation is being used for various species (43). Under natural conditions, the life span of fresh coconut pollen is only a few days (34). Coconut pollen can be conserved for short periods by freeze-drying and storage at room temperature or in a refrigerator (36, 44). The reports on coconut pollen cryopreservation showed that liquid nitrogen (LN) storage for 24 h did not affect *in vitro* germination (5, 24).

At ultra low temperature storage most of the molecular reactions in the cells are retarded and viability may extend to hundreds of years. But in some plants like *Hevea*, pollen viability declined from 20% *in vitro* germination after 1 month to 2% after 5 months LN storage (18).

Hence, before coconut pollen cryostorage can be routinely employed as a complementary technique to field conservation, the effect of various parameters, including the period of pollen collection and the LN storage duration, on viability and fertility and the genotypic effect have to be evaluated. In this study, pollen of two coconut varieties [West Coast Tall (WCT) and Chowghat Orange Dwarf (COD)] was collected during the months of March, April and May over three consecutive years and tested for germination after LN exposure. Pollen germination of these two varieties was measured after cryostorage periods between 24 h and 6 years. Hand-pollination of female flowers was performed using WCT and COD pollen cryostored for 6 months and 4 years and seed set was measured. Finally, the cryopreservation protocol was validated by measuring the *in vitro* germination of pollen of five Tall and five Dwarf coconut accessions.

MATERIALS AND METHODS

Plant materials

To test the effect of cryopreservation and of cryostorage duration on pollen germination and fertility, five WCT and COD palms were selected randomly from the palms used as male parents for hybridization studies at the Central Plantation Crops Research Institute, Kasaragod, Kerala (India).

Pollen collection

Pollen was collected from WCT and COD palms from 2007 to 2009 in March, April, and May to evaluate the effect of pollen collection period on cryopreservation. Pollen from these palms was also used to evaluate the effect of LN storage duration on germination and on seed set between 2007 and 2013. After the complete emergence from the leaf axils (once every 20-25 days)

and just before the natural opening of the male flowers, inflorescences were bagged to prevent contamination by foreign pollen. After 6 to 7 days, spikes were collected on a bright sunny day between 8 – 10 am when the male flowers started opening from the tip. Male flowers were stripped off from the spikes and placed in Petri dishes lined with aluminium foil, which were stored for 24 h in an oven set at 40ºC (44). The dried male flowers were sieved (mesh size - 0.2 mm) to collect pollen for cryopreservation experiments and also for testing the moisture content using moisture analyzer (A & D Company Ltd, Japan, MF 50) set at 70ºC and the moisture content was found to be 7.5% after a period of 11 months.

Pollen cryopreservation procedure

The desiccated pollen (20 mg) was wrapped in aluminium foils, which were inserted in 2 ml cryovials and plunged directly in LN. The vials were retrieved from cryogenic storage after different periods (24 h, 6 months, 1, 1.5, 2, 3, 4 and 6 years). For rewarming, cryovials were placed at room temperature for 1 h. Pollen was then used for viability testing by *in vitro* germination or for controlled hand-pollination in the field.

In vitro *pollen germination*

For germination of non-cryopreserved and cryopreserved pollen, the medium employed consisted of 8% sucrose, 1% agar, 1% gelatin and 0.01% boric acid (25). A microscopic slide with pollen evenly distributed on the medium surface was incubated in a Petri dish lined with moist filter paper at 32 ± 2ºC for germination. Pollen germination was observed under the microscope after 90 min incubation. Pollen grains were considered to have germinated when pollen tube length (PTL) was at least equal to or greater than the pollen grain diameter (21). A minimum of 400-500 pollen grains were counted in 10 randomly selected microscopic fields for measurement of germination percentage. Photographs of each field were taken using a Leica camera (DFC 250). Pollen tube length was measured using the Leica Q Win software. Mean pollen tube length was calculated as the average length of 100 to 150 pollen tubes in 10 randomly selected microscopic fields.

Fertility assessment of cryostored pollen

Pollen from WCT and COD palms cryostored for 6 months and 4 years, respectively was utilized for pollination experiments. After rewarming, cryopreserved pollen was mixed with neutral talc (1:9 ratio) and used for pollination following the standard CPCRI procedure (7). One palm each of WCT and COD with a minimum of 25 female flowers was selected for fertility studies using pollen cryopreserved for different durations. The pollinated bunches were marked by giving an unique number to each pollination. The percentage of nut set in each cross was recorded. Cryostored pollen from WCT palms was utilized for crosses with COD palms as the seed parent and vice versa. The resultant nuts were harvested, embryos were excised, inoculated *in vitro* and their germination was tested using the CPCRI embryo culture protocol (26).

The cryopreservation protocol developed was validated in 2010 using pollen from five Tall [Laccadive Ordinary Tall (LCT); Andaman Giant Tall (AGT); Philippine Ordinary Tall (PHOT); Java Tall (JVT); Cochin China Tall (CCNT)] and five Dwarf accessions [Malayan Yellow Dwarf (MYD); Cameroon Red Dwarf (CRD); Kenthali Orange Dwarf (KTOD); Gangabondam Green Dwarf (GBGD); Chowghat Green Dwarf (CGD)].

Statistical analyses of data

The results of viability tests (*in vitro* pollen germination and pollen tube length) were subjected to one-way ANOVA and significant differences were evaluated by DMRT using the SPSS 15.0 software.

RESULTS

Effect of pollen collection period on cryopreservation

After dusting pollen on germination medium, pollen started swelling and produced tubes within 30 min, whereas none of the pollen germinated without rehydration in humid environment in the vicinity of the pollen. Different stages of pollen germination are shown on Fig. 1a and b. Germination and PTL of desiccated and cryopreserved pollen were not significantly different for both WCT and COD over the three harvest months of the three consecutive years of study (Table 1). Pollen germination ranged from 24 to 32% in desiccated pollen whereas it was between 26 and 29% in cryopreserved COD pollen. In case of WCT, germination ranged from 30 to 31% in desiccated pollen, while it was between 28 and 32% in cryopreserved pollen. PTL of cryopreserved pollen ranged between 224-390 μm and 226-396 μm for COD and WCT, respectively.

Effect of cryostorage duration on viability and vigour of pollen

There was no effect of LN storage duration on germination of WCT pollen (Table 2). In the case of COD, germination was similar for all LN storage durations tested except for 1.0 and 1.5 years, where it was significantly higher. However, significant cultivar*LN storage duration interaction was observed for pollen germination. Among the two cultivars, WCT pollen cryostored for 1.5 years showed significantly higher germination compared to all other storage durations studied (P=0.001). There was no effect of LN storage duration on COD PTL. By contrast, in case of WCT, PTL was significantly higher after 1.5 years LN storage compared to other storage durations as well as between cultivars (*P*=0.0001).

The percentage of pollen with different

Table 1. Effect of desiccation (-LN) and of cryopreservation (+LN) on germination (G, %) and pollen tube length (PTL, μm) of COD and WCT pollen collected in March-May during three consecutive years (2007-09).

Harvest month	COD G (%)		WCT G (%)		COD PTL (μm)		WCT PTL (μm)	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
March 2007	26.7 ± 9.8	30.0 ± 10.8	29.0 ±13.6	33.4 ±10.9	249.0 ±46.7	271.0 ±49.6	249.8 ±56.6	253.8 ±31.3
April 2007	21.4 ±13.4	28.8 ±5.0	34.9 ±8.1	25.1 ±12.6	240.1 ±106.2	358.3 ±41.4	351.8 ±68.0	313.8 ±86.0
May 2007	23.8 ±7.6	20.1 ±11.9	29.6 ±12.7	31.7 ±114.3	249.3 ±5.9	223.5 ±69.4	280.6 ±89.9	286.5 ±118.5
March 2008	25.2 ±7.0	26.2 ±8.5	29.3 ±5.4	29.7 ±88.9	261.9 ±40.8	255.0 ±40.8	334.4 ±19.6	294.4 ±107.2
April 2008	39.3 ±13.1	36.0 ±10.8	37.4 ±15.0	29.2 ±15.4	375.1 ±65.7	390.0 ±43.5	408.0 ±128.6	396.2 ±174.1
May 2008	32.9 ±6.0	25.7 ±11.4	26.9 ±5.5	26.4 ±5.9	411.2 ±67.4	372.7 ±87.2	390.9 ±52.9	381.0 ±13.7
March 2009	27.5 ±6.4	28.0 ±4.2	31.6 ±10.6	35.3 ±12.5	376.6 ±165.5	319.6 ±107.7	366.1 ±143.9	342.3 ±131.1
April 2009	27.7 ±6.3	21.3 ±5.8	20.9 ±3.3	24.7 ±5.7	328.8 ±117.4	364.3 ±104.8	249.7 ±40.5	225.5 ±59.3
May 2009	42.2 ±19.7	34.7 ±6.8	38.8 ±11.7	34.4 ±8.5	367.2 ±107.7	369.1 ±188.2	456.44 ±89.3	293.2 ±59.5
	P = 0.90		P = 0.62		P = 0.80		P = 0.52	

P represents the probability values between desiccated and cryopreserved pollen samples

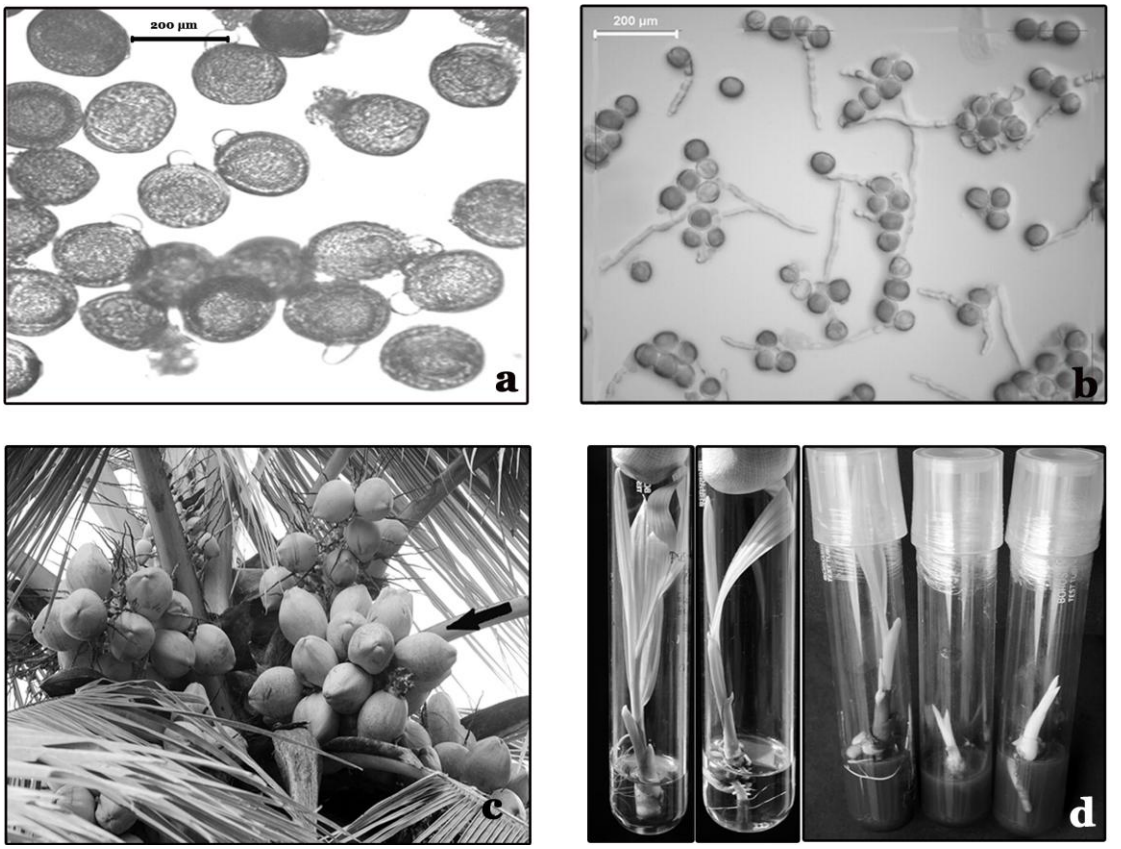

Figure 1. Different stages of viability / fertility studies using desiccated / cryopreserved coconut pollen: (a) Protrusion of pollen tube after 30 min incubation; (b) Germination of pollen cryostored for 6 years; (c) Nut set in COD palm using WCT pollen cryopreserved for 4 years; (d) Embryo cultured plantlets from hybrid nuts produced using cryopreserved pollen.

PTL ranges was low in pollen cryopreserved for 6 months, 1.5, 3 and 4 years but the maximum PTL exceeded that of oven-dried pollen (Fig. 2). For other durations (24 h and 1 year) pollen with different ranges of PTL were found in low percentage compared to other conditions. There was no interaction between storage duration and range of PTL in cryopreserved WCT pollen.

In the case of COD pollen, the storage period did not influence PTL over the interval studied (Table 2). In oven-dried pollen from COD palms, even though the percentage of fast growing pollen was high,

Table 2. Effect of LN storage duration on germination (G, %) and pollen tube length (PTL, µm) of COD and WCT pollen. In columns, values (±SD) followed by the same letter are not significantly different (p = 0.05).

LN storage duration	COD		WCT	
	G (%)	PTL (µm)	G (%)	PTL (µm)
0 (Oven dried)	33.5 ± 10.0^{b}	257.7 ± 49.6^{a}	33.3 ± 2.7^{a}	239.1 ± 12.7^{cde}
24 h	32.6 ± 7.6^{b}	257.7 ± 117.0^{a}	32.1 ± 2.7^{a}	228.6 ± 16.2^{de}
6 months	29.4 ± 7.1^{b}	207.1 ± 126.2^{a}	33.3 ± 2.7^{a}	193.2 ± 11.0^{e}
1 year	44.1 ± 10.2^{a}	213.7 ± 29.7^{a}	32.2 ± 2.7^{a}	424.5 ± 22.3^{ab}
1.5 y	44.1 ± 10.2^{a}	204.4 ± 85.6^{a}	40.1 ± 2.7^{a}	436.6 ± 23.1^{a}
3.0 y	30.1 ± 5.1^{b}	220.1 ± 113.5^{a}	34.0 ± 2.7^{a}	250.0 ± 13.2^{cd}
4.0 y	29.3±7.2^{b}	228.8±41.6^{a}	38.1 ± 2.5^{a}	376.7 ± 16.2^{b}
6.0 y	-	-	38.2 ± 2.5^{a}	283.0 ± 12.0^{c}

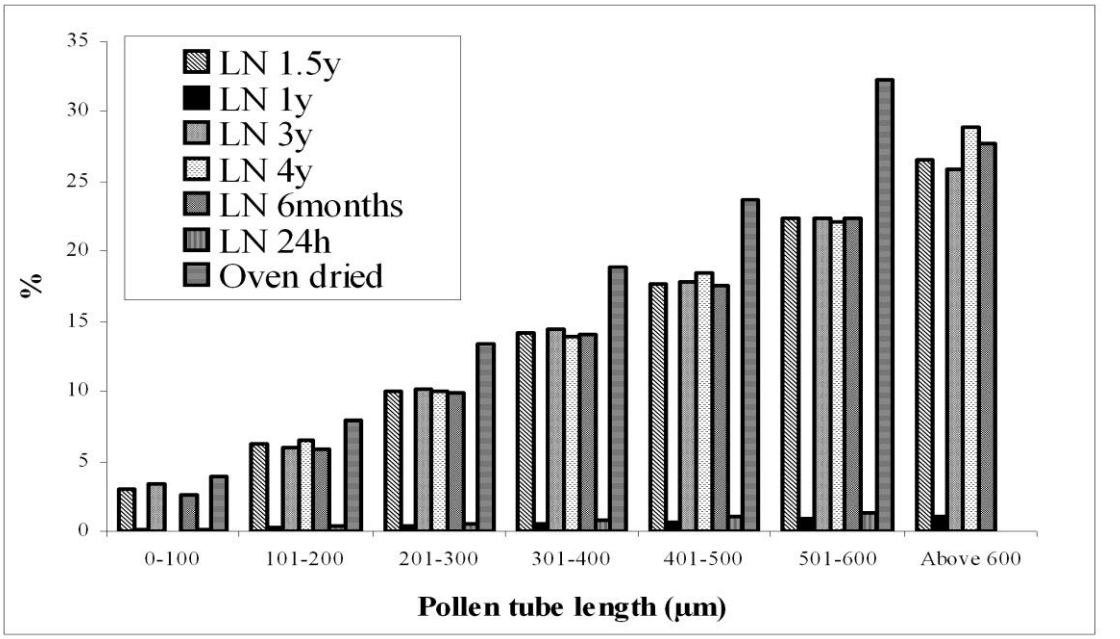

Figure 2. Percentage of pollen having diverse tube lengths (µm) from pollen samples (WCT) cryostored for different periods.

it had a shorter length compared to pollen cryostored for 6 months, 1.5 and 3 years (Fig. 3).

Fertility of cryopreserved pollen

Pollen cryostored for 4 y could be stored for up to 4 d at room temperature after rewarming without any significant decrease in germination and PTL in case of WCT, whereas in COD significant differences were noticed after day one (Table 3). Both parameters decreased dramatically after longer storage durations at room temperature.

Fertility of COD and WCT pollen was slightly higher after 6 months cryostorage compared to 4 years cryostorage (Table 4). Seed set after hand-pollination using cryostored pollen was found similar to that produced under natural conditions with palms selected for the study (Figs. 1c). One hundred percent germination was observed

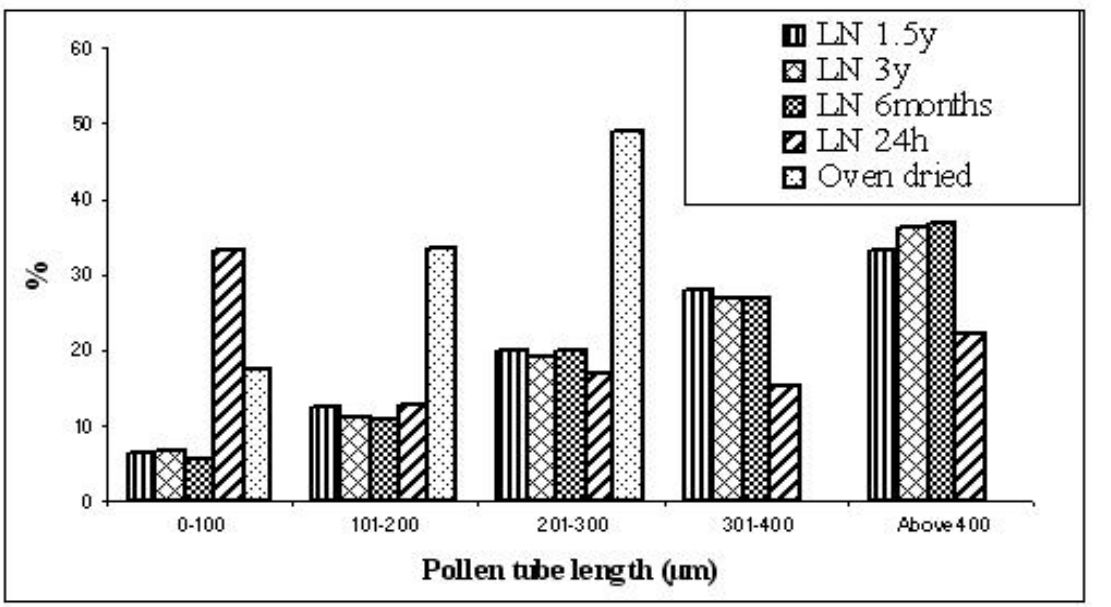

Figure 3. Percentage of pollen having different tube lengths (µm) from pollen samples (COD) cryostored for different periods.

Table 3. Effect of storage period (days) at ambient temperature after rewarming on germination (G, %) and pollen tube length (PTL, µm) of varieties (var) WCT and COD pollen cryostored for 4 years. In rows, values (±SD) followed by the same letter are not significantly different (P = 0.0001).

Var		Storage period after rewarming (days)					
		1	2	3	4	5	7
WCT	G (%)	30.1 ±5.1^{A}	34.2 ±5.2^{A}	29.4 ±4.5^{A}	29.7 ±9.5^{A}	17.7 ±3.6^{B}	13.1 ±5.8^{B}
	PTL (µm)	224.3 ±36.2^{A}	234.23 ±27.4^{A}	217.96 ±27.4^{A}	231.25 ±26.0^{A}	135.61 ±15^{B}	124.43 ±10.3^{B}
COD	G (%)	34.0 ±6.8^{A}	26.2 ±4.2^{B}	29.5 ±4^{AB}	27.4 ±10^{B}	15.4 ±6^{C}	12.8 ±3^{C}
	PTL (µm)	250.6 ±38.6^{A}	209.6 ±42.0^{B}	225.71 ±35.2^{AB}	229.26 ±45.7^{AB}	140.87 ±28.5^{C}	112.47 ±14.8^{C}

in embryos extracted from hybrid nuts produced with cryostored pollen and plantlet development was normal (Fig.1d).

Cryopreservation of pollen of Tall and Dwarf accessions

In case of Tall accessions, there were no significant differences in germination of desiccated (-LN) and cryopreserved (+LN) pollen within and between accessions (Table 5). However, significant differences in PTL were observed between accessions, PHOT showing higher PTL values and CCNT lower values compared to the other three accessions. LN exposure significantly decreased PTL for three (AGT, PHOT and LCT) out of the five accessions tested.

In Dwarf palms, germination of desiccated pollen was significantly higher in MYD and CRD compared to CGD and CBGD (Table 5). After cryopreservation, germination was significantly higher in MYD compared to the other accessions. Cryopreserved pollen displayed significantly higher germination in CGD, CBGD and MYD compared to desiccated pollen. PTL was significantly higher in CRD and CGD accessions compared to the other three accessions. After cryopreservation, CRD PTL was significantly lower compared to the other four accessions. Cryopreservation significantly decreased PTL in the case of CRD.

DISCUSSION

Pollen collection and processing are of critical importance for the establishment of a pollen bank (20). In coconut, maximum pollen production in individual male flowers as well as in the inflorescences occurred

Table 4. Germination (G, %) and fertility (seed set, %) of WCT and COD pollen cryostored for 6 months and 4 years.

LN storage period	Pollen parent	Seed parent	G (%)	Seed set (%)
6 months	WCT	COD	33.3	28.0
6 months	COD	WCT	29.4	20.5
4 years	WCT	COD	44.3	20.0
4 years	COD	WCT	39.7	12.5

Table 5. *In vitro* germination (G, %) and pollen tube length (PLT, µm) of desiccated (-LN) and cryopreserved (+LN) pollen of five Tall and five Dwarf coconut accessions. In rows, values followed by different lower case letters indicate significant differences within accessions (p = 0.05). In columns, values followed by different upper case letters indicate significant differences between accessions (P = 0.05).

	G (%)		PLT (µm)	
Accession	-LN	+LN	-LN	+LN
Tall				
AGT	23.58 ± 1.9$^{a\ A}$	24.7 ± 3.3$^{a\ A}$	285.2 ± 15.47^{aB}	229.8 ± 18.5$^{b\ C}$
JVT	24.92 ± 1.9$^{a\ A}$	24.1 ± 1.8$^{a\ A}$	287.9 ± 12.86$^{a\ B}$	282.3 ± 10.3$^{a\ B}$
PHOT	27.68 ± 1.9$^{a\ A}$	27.2 ± 1.8$^{a\ A}$	503.5 ± 12.98$^{a\ A}$	340.2 ± 9.9$^{b\ A}$
CCNT	26.22 ± 1.9$^{a\ A}$	30.5 ± 1.8$^{a\ A}$	188.4 ± 13.94$^{a\ C}$	186.6 ± 9.3$^{a\ D}$
LCT	31.81 ± 1.9$^{a\ A}$	30.9 ± 1.8$^{a\ A}$	298.8 ± 11.85$^{a\ B}$	207.9 ± 8.6$^{b\ CD}$
Dwarf				
CRD	29.2 ± 2.8$^{a\ A}$	31.6 ±3.1$^{a\ B}$	310.4 ± 12.9$^{a\ A}$	201.4 ± 9.3$^{b\ C}$
CGD	19.3 ± 2.8$^{b\ B}$	31.1 ± 4.9$^{a\ B}$	307.3 ± 18.8$^{a\ A}$	289.9 ± 21.4$^{a\ A}$
GBGD	19.4 ± 2.8$^{b\ B}$	25.2 ±3.1$^{a\ B}$	222.2 ± 19.6$^{a\ C}$	259.6 ± 10.8$^{a\ AB}$
KTOD	27.3 ± 2.8$^{a\ AB}$	29.8 ± 3.1$^{a\ B}$	236.3 ± 15.3$^{a\ BC}$	224.8 ± 12.4$^{a\ B}$
MYD	35.6 ± 2.8$^{b\ A}$	48.9 ± 3.1$^{a\ A}$	281.3 ± 17.4$^{a\ AB}$	289.2 ± 12.2$^{a\ A}$

during the summer season starting from February to May (17). Hence this study was undertaken to verify the effectiveness of pollen collection, processing and cryopreservation during the summer months. It is also prudent to collect pollen during periods of low relative air humidity, during which it can be expected that the amount of water to extract from pollen before its cryopreservation will be lower (3). Water content of pollen grains at the time of dispersal varies among different plant families in the range of 15 to 35% FW (19). In the majority of cases, partial desiccation of pollen is necessary to retain its viability after LN storage (2). Drying of coconut male flowers in the oven at 40 ºC for 24 h reduced MC to 7.5%, which was crucial for maintaining its viability and fertility after LN exposure. Indeed, *in vitro* germination was nil when pollen was cryopreserved without previous drying. In tomato, desiccation of pollen to 6.8 to 9.3% MC prior to cryogenic storage enhanced its germination ability (56.8 to 57.4%) compared to fresh pollen with 10.6% MC for which germination percentage was lower (52.7%) (24). Excessive pollen dehydration before cryopreservation may lead to viability loss as reported with *Rosa* and *Dioscorea* species (11). Cooling and warming rates have been shown to be critical in preserving enzymatic activity of cryogenic stored pollen (29). Sedgley (37) reported that rapid cooling of *Persea* pollen was successful because there was less possibility of ice crystal damage compared to cooling at a lower rate. In the present study, rapid cooling combined with slow warming at room temperature had no adverse effect on coconut pollen ability to germinate *in vitro*.

Pollen germination *in vitro* is routinely used for assessment of pollen viability and it is often correlated with its fertilizing ability (1). In agreement with Karun *et al*. (25), coconut desiccated or cryopreserved pollen collected from WCT and COD palms germinated well on medium containing sucrose and boric acid under high humidity. It was also observed that rehydration of partially desiccated coconut pollen was necessary for its germination. Similar observations were made earlier in coconut (4) and in tomato pollen (24). There are also other reports stating that hydration of dried pollen results in an overall improvement in germination (8, 9), which suggests that inadequate hydration may result in a false indication of low viability. While mature coconut pollen is tricellular, it behaved like bicellular pollen in its response to desiccation and *in vitro* germination. Franchi

et al. (16) classified coconut as having recalcitrant seeds and orthodox pollen. Even though *in vitro* pollen germination has been reported to be difficult in tricellular pollen (40) coconut pollen germinated easily on a simple medium supplemented with sucrose and boron.

Pollen of other palm species including date palm (27) and oil palm (42) has been successfully cryopreserved. The coconut pollen cryopreservation protocol was developed by Anitha *et al.* (25) utilizing nine palms from WCT variety of coconut. Germination of cryopreserved pollen from these palms was found to be significantly less compared to fresh pollen but on par with incubator-dried pollen. Moreover overall pollen tube length was significantly higher when pollen were incubator-dried (190.8µm) or cryopreserved (192.5µm) compared to fresh pollen (140.8µm). The results of the present study are consistent with the earlier report (25). In this work, the retention of WCT and COD cryopreserved pollen viability above a mean value of 25% over three consecutive years confirmed the high potential of pollen to contribute to long-term conservation of coconut germplasm.

Germination of pollen is expected to remain stable for extended cryostorage durations. However, there are contradictory reports of modifications of pollen viability after cryostorage. *In vitro* germination of *Hevea* pollen decreased from 20% after 1 month to 2% after 5 months LN storage (18). By contrast, in some *Prunus mume* cultivars, pollen germination increased significantly after 1-4 years cryostorage compared to fresh pollen without storage (45). Such changes may be due to differences in pollen sample preparation, resulting in different MCs and/or to difficulties in assessment of pollen viability, for example (41). Over the cryogenic storage periods investigated in the present study (up to 6 years in case of WCT), no significant changes were seen in pollen germination of the two coconut varieties studied.

Assessment of pollen vigour in addition to pollen germination is essential for *in vitro* pollen germination studies since viability and vigour are considered as important factors influencing gametic selection in the style (32). In *Nicotiana tabacum* and *Tradescantia virginiana,* pollen storage stress resulted in a reduction in pollen tube vigour before any reduction in its viability (41). The results of the present study indicate that vigour of pollen stored in LN, as assessed by mean as well as maximum pollen tube length, improved over the years of storage. In *Crotalaria* and *Brassica*, there was no reduction in pollen tube vigour when storage was confined to a limited period of 7 days for *Crotalaria* and 16 days for *Brassica* (28). But beyond the optimal period, the stored pollen showed a significant reduction not only in germination percentage but also in tube length. A correlation between pollen tube growth speed and weight of the resultant seeds and seedlings has been reported in *Zea mays* pollen (12, 13). Studies by Ottaviano *et al*. (30) have shown that the progeny derived from pollen selected for faster tube growth resulted in increased seedling vigour, kernel weight, and root growth *in vitro*.

Pollen germination is routinely used to assess viability of cryopreserved pollen. However, the ultimate proof of pollen cryopreservation efficiency should be the production of viable seeds after fertilization of female flowers with pollen cryostored for various durations. In mango and litchi (6), successful seed set was observed by using pollen cryostored for 4 years. *Gladiolus* pollen cryostored for 10 years retained high levels of *in vitro* germination and seed set (35). Sugar beet pollen stored for 17 years in LN was able to produce viable seeds (33). By contrast, cryostored broccoli pollen displayed high viability but the seeds produced exhibited reduced vigour and rapidly lost germinability (10). In our experiments, the formation of viable mature seeds after hand-pollination of receptive female flowers with coconut pollen cryostored for 6 months and 4 years

confirmed the efficiency of the established cryopreservation protocol.

It has been observed in *Prunus mume* pollen that environmental conditions at the time of pollination may affect seed set (46). In case of coconut, our experiments showed that viability of cryostored coconut pollen was maintained for 4 days after rewarming. This will allow the maximal use of cryostored pollen as hand-pollination can be performed over the few days of the receptive stage of female flowers in inflorescences and can be delayed in case of adverse weather conditions.

Differences in cryopreserved pollen germination and PTL were observed among the Tall and Dwarf coconut accessions studied. The differences noted in germination and PTL of different *Prunus* spp. reflected its genotypic variability (23). Differences in germination and PTL between cultivars and genotypes have been reported in a number of species including peach, plum, prune, sour and sweet cherry, almond and apricot (38, 39). Studies have shown that pollen cytoplasmic carbohydrates and sucrose are involved in protecting pollen viability during exposure and dispersal (31). The genotypic differences in germination and PTL in cotton pollen could be due to differences in pollen carbohydrate concentration (22). Further studies are thus needed to investigate the differences in pollen germination and PTL of the Tall and Dwarf coconut accessions employed in this study.

The present study is the first report of successful long-term cryostorage of coconut pollen. *In vitro* germination and fertility of pollen of two coconut varieties remained unchanged after 4 years LN storage. This work confirmed that trinucleate coconut pollen is tolerant to desiccation and LN exposure and that its viability can be easily assessed by *in vitro* germination on a simple medium enriched with sucrose and boric acid. Pollen can be efficiently collected during its maximum production period (March-May), partially desiccated and cryostored. The establishment of pollen cryobanks to complement field collections will significantly improve the conservation of coconut genetic resources and contribute to further breeding efforts.

REFERENCES

1. Aloni B, Mary P, Mason P & Leah K (2001) *Physiol. Plant*. **112**, 505–512.
2. Barnabas B & Rajki E (1981) *Ann. Bot.* **48,** 861-864.
3. Barnabas B & Fridvalszky L (1984) *Acta Bot. Hungary* **30**, 329 332.
4. Bernard G (1973) *Oléagineux* **28,** 447-551.
5. Caroline de A M, Catrine RFM, Eurico EPL, Semíramis RRR, Francisco ER, & Ana da SL (2014) *Acta Scient. Agron.* **36**, 227-232.
6. Chaudhury R, Malik SK & Rajan S (2010) *CryoLetters* **31**, 268-278.
7. CPCRI (2009) Technical Bulletin No. 57.
8. Crowe JH, Hoeskstra FA & Crowe LM (1989) *Proc. Nat. Acad. Sci.* **86**, 520-525.
9. Crowe JH, Hoeskstra FA, Crowe LM, Anchordoguy T J & Drobnis E (1989) *Cryobiology* **26**, 76-84.
10. Crisp P & Grout WW (1984) *Euphytica* **68**, 77-84.
11. Daniel I, Ng NO, Tayo TO & Togun AO (2002) *J. Agr. Sci.* **138**, 57-62.
12. David LM (1971) *Science* **171**, 1155-1156.
13. David LM (1974) Nature **249**, 491-492.
14. Engelmann F (1997) in *Biotechnology and Plant Genetic Resources: Conservation and Use*, (eds) BV Ford-Lloyd, JH Newbury & JA Callow. CABI, Wallingford, pp 119-162.
15. Engelmann F, Assy-Bah B, Bagniol S, Dumet D & Michaux-Ferrière N (1995) in *Biotechnology in Agriculture and Forestry, Vol 32, Cryopreservation of Plant Germplasm I*, (ed) YPS Bajaj, Springer-Verlag, Berlin Heidelberg, pp 148-169.
16. Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM & Pacini, E (2011) *J. Exp. Bot.* **62**: 5267-81.

17. Gangolly SR, Kamalakaran AK, Balakrishnan TK & Pandalai KM (1961) *Ind. Coco. J.* **14**, 49-66.
18. Hamzah & S Leene C (1986**)** *J. Nat. Rubber Res.* **11**, 115-204.
19. Heslop-Harrison J (1979) *Amer. J. Bot.* **66**, 737-743.
20. Hoekstra FA (1995) In *Collecting Plant Genetic Diversity. Technical Guidelines*, (ed) Guarino L, Rao VR & Reid R, CAB International, pp. 527-550.
21. Kakani VG, Prasad PVV, Craufurd PQ & Wheeler TR (2002) *Plant, Cell & Environ.* **25**, 1651–1661.
22. Kakani VG, Reddy KR, Koti S, Wallace TP, Prasad PVV, Reddy VR & Zhao D (2005) *Ann. Bot.* **96**, 59-67.
23. Karim S, Behrouz S, Vahid R & Mahmood K (2011) *Trees* **25**, 809–822.
24. Karipidis C, Olympios C, Passam HC & Savvas D (2007) *J. Hortic. Sci. Biotech.* **82**, 29-34.
25. Karun A, Sajini KK, Meera N, Kumaran PM & Samsudheen K (2006) *J. Plantation Crops* **34**, 568-571.
26. Karun A, Sajini KK & Shivashankar S (1999) *Indian J. Hortic.* **56**, 348-353.
27. Mortazavi SMH, Arzani K & Moieni (2010) *J. Agr. Sci. Tech.* **12**, 181-189.
28. Nandakumar PBA, Chaudhury R & Shivanna KR (1988) *Curr. Sci.* **57,** 557-559.
29. Nath J & Anderson JO (1975) *Cryobiology* **12**, 81-88.
30. Ottaviano E, Sari-Gorla M & Villa M (1988) *Theor. Appl. Genet.* **76**, 601- 608.
31. Pacini E (1996) *Sex. Plant Reprod.* **22**, 362-366.
32. Page T, Moore GM, Will J & Halloran GM (2006) *Austral. J. Bot.* **54**, 553-558.
33. Panella L, Wheeler L & McClintock ME (2009) *J. Sugar Beet Res.* **46,** 1-9.
34. Patel JS (1938) *The Coconut - A Monograph*. Govt. Press, Madras. pp.90-133.
35. Rajasekharan PE, Rao TM, Janakiram T & Ganeshan S (1994) *Euphytica* **80**, 105-109.
36. Rognon F & Nucé de Lamothe M (1978) *Oléagineux* **33**, 17-21.
37. Sedgley M (1981) *Euphytica* **30**, 595-599.
38. Sharafi Y & Bahmani A (2011) *J. Med. Plant Res.* **5**, 266-269.
39. Sharafi Y & Bahmani A (2011) *J. Med. Plant Res.* **5**, 275-279.
40. Shivanna KR & Johri BM (1985) *The Angiosperm Pollen: Structure Function.* Wiley Eastern Publishing, New Delhi.
41. Shivanna KR, Linskens HF & Cresti M (1991) *Theor. Applied Genet.* **81**, 38-42.
42. Tandon R, Chaudhury R & Shivanna KR (2007) *Curr. Sci.* **92**, 182-183.
43. Towill LE & Walters C (2000) in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, (eds) Engelmann, F and Takagi H, JIRCAS, Tsukuba, Japan / IPGRI, Rome, Italy, pp 115-129.
44. Whitehead RA (1963) *Euphytica* **12**, 167-177.
45. Withers LA & Engels JMM (1990) *IBPGR Newsletter for Asia and the Pacific* 3, 1-2.
46. Zang YL, Chen RD, Huang CJ & Liu Y (2009) *CryoLetters* **30**, 165-170.