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CRYOPRESERVATION OF ARECANUT (*Areca catechu* L.) POLLEN

Anitha Karun[1], K.K. Sajini[1*], K.S. Muralikrishna[1], M.K. Rajesh[1] and Florent Engelmann[2]

[1]ICAR-Central Plantation Crops Research Institute, Kasaragod 671124, Kerala, India
[2]IRD, UMR DIADE, BP 64501, 34394 Montpellier, Cedex 05, France
*Corresponding author email: kk.sajini@gmail.com

Abstract

BACKGROUND: Cryopreservation opens new avenues in the field of genetic resource conservation, especially in recalcitrant seeded palms such as arecanut for which field genebanks are exposed to pest and disease attacks and natural calamities. It is only through cryopreservation that the safety of the conserved germplasm can be assured at a relatively low cost for extended periods. **OBJECTIVE:** The objective of this work was to standardize various aspects of arecanut pollen cryopreservation, viz. collection and desiccation of pollen, *in vitro* germination, viability and fecundity studies. **MATERIALS AND METHODS:** Pollens of three arecanut genotypes (Sumangala, Hirehalli Dwarf and Hirehalli Dwarf x Sumangala) were collected in December 2013-February 2014. *In vitro* viability tests were conducted using fresh and desiccated pollen. Desiccated pollen was cryopreserved by direct immersion in liquid nitrogen and cryostored for different durations (24 hours to 2 years). Viability and fertility studies were conducted using cryopreserved pollen. **RESULTS:** Pollen extraction was achieved from fully opened male flowers by desiccation at room temperature (33-34°C). A medium containing 2.5 g/L sucrose was found to be best for *in vitro* germination at room temperature. There was no significant difference in germination between desiccated and cryopreserved pollen whereas pollen tube length decreased significantly after cryopreservation. Fertility studies using HD x Sumangala pollen cryostored for various durations (1 month, 1 year and 2 years) showed the setting of 70, 43 and 62%, respectively. Normal nut set was observed using cryopreserved pollen. **CONCLUSION:** Pollen cryopreservation is a viable option for germplasm conservation and hybridization programmes in arecanut.

Keywords: *Areca catechu* L., arecanut, pollen, cryopreservation, germination, vigor, fertility

INTRODUCTION

Arecanut (*Areca catechu* L. Arecaceae) is commonly referred to as betel nut and grows in the tropical Pacific, Asia, and parts of East Africa. The genus *Areca* consists of 76 species (20), of which *Areca catechu* is the only cultivated one. Arecanut is an allotetraploid with a chromosome number of 2n = 32 and is highly cross-pollinated. India ranks first in the world for production of arecanut (7). In India, arecanut is mainly cultivated in the states of Assam, Karnataka, Kerala, Maharashtra, Tamilnadu, Goa, West Bengal and Tripura. Arecanut forms an essential requisite for several religious and social ceremonies and its use dates back to Vedic period. According to 'Vagbhatta', an ancient medical text, arecanut has its use against leucoderma, leprosy, cough, epilepsy, worms and obesity (1). Being a highly valued commercial crop, its contribution to the national economy in terms of livelihood, employment and income is significant. The highly varied uses of nuts as well as other parts of the palms like husk fibers, stem, timber, tannins, leaves, etc. makes the tree very remunerative for farmers.

Arecanut seeds are classified as recalcitrant (28). Arecanut genetic diversity is maintained as live collections in field genebanks. In India, arecanut germplasm is mainly maintained by ICAR-Central Plantation Crops Research Institute (RS) at Vittal, Karnataka State. In the

present scenario of devastating diseases and changing environmental conditions and urbanization, other alternative approaches, such as *in vitro* conservation of genetic diversity, is gaining momentum. Pollen is a useful source of diverse alleles within gene pools and conserving pollen has opened up new avenues for the creation of new varieties by breeders. Pollen of many species is desiccation tolerant, *i.e.*, it can be dried to moisture contents (MC) below 5% on a dry weight (DW) basis (33), but it is difficult to maintain its viability over long periods at ambient temperature or under refrigerated conditions. Hence pollen cryopreservation [liquid nitrogen (LN), -196°C] is the only option for long-term conservation, enabling its utilization after extended storage periods.

Cryopreservation of pollen is also beneficial for carrying out investigations on both fundamental and applied aspects of pollen biology. It is also beneficial for making interspecific hybrids in some crops which flower at different periods like *Panax* (36). The international transfer of germplasm in the form of dry pollen is generally not restricted (11). Arecanut pollen remains viable for a few hours (8-9 h) only (25) and its production is restricted to a few months per year (October to February). Hence, in addition to long-term conservation, arecanut pollen cryostorage would be beneficial not only for extending its viability but also for use in hybrid seed production or in assisted pollination for increasing yield. Quality pollen from good pollen parents with high combining ability collected during its peak production period could be stored for hybrid seed production, without having to wait for the original palms to flower during consecutive years.

The present study was carried out to standardize various steps of the arecanut pollen cryopreservation protocol, *viz.* desiccation of male flowers, pollen collection, *in vitro* germination and viability and fecundity studies.

MATERIALS AND METHODS

Plant material

Three palms (8-10 years old) each from three arecanut genotypes (Sumangala, Hirehalli Dwarf, Hirehalli Dwarf x Sumangala) maintained at ICAR-CPCRI, Kasaragod, Kerala State, India were utilized for the experiments.

Pollen collection

The spikes with staminate (male) flowers were excised from fully opened inflorescences between 9-10 AM during the months of December 2013 to February 2014. Fresh pollen was extracted by gently tapping fully opened staminate flowers (Fig. 1b). For collection of desiccated pollen, opened flowers and flowers expected to open the next day were separated from intact spikes and placed on a piece of dry paper and gently crushed to make the flowers fully opened. These flowers were placed on aluminium foils in Petri dishes and dried either in incubators set at two different temperatures (30 and 32°C) or at room temperature (33-34°C) for 24 h down to 6-7% MC to study the effect of drying on pollen collection. After drying of male flowers, pollen was collected using a sieving apparatus fitted with 0.2 mm pore size mesh (Fig. 1d). For moisture determination pre-weighed desiccated pollen was kept in an oven at 100°C for 24 hours.

In vitro germination

For standardization of arecanut pollen germination medium, three media containing 0.01% boric acid, 1% agar and gelatin and different sucrose concentrations (2.5, 3.0 and 4.0%) were compared. Freshly prepared medium was spread uniformly over a slide for cooling. Fresh or desiccated/cryopreserved pollen grains were evenly distributed over the slide and incubated at room temperature (33 to 34°C) for a period of 90 min in a Petri dish lined with moist filter paper, closed to maintain high humidity. The germination percentage was calculated by counting the germinated pollen in three randomly selected fields on the slide with a minimum of 30-50 pollen grains per field. Pollen grains were considered viable if tube growth exceeded pollen grain diameter and germination percentage was calculated. The length of pollen tubes was measured using the Leica Qwin software with 60-80 pollen tubes in 10 randomly selected fields and average was calculated. This procedure was followed in all further studies.

In order to test the effect of temperature on pollen *in vitro* germination, the slides containing pollen dusted on germination medium were placed in incubators set at 30 and 32°C or at room temperature (RT, 33-34°C) for 90 min. Since the highest germination % was obtained with medium containing 2.5% sucrose, it was used as standard medium for further studies. The

viability of pollen stored at RT for 5 days under desiccated conditions was also studied to evaluate the viability loss during storage. Germination percentage was calculated from day 1 to day 5.

Pollen cryopreservation procedure

Desiccated pollen grains (0.4-0.5 g FW) were wrapped in aluminum foils, which were inserted in cryovials, directly plunged in LN and cryostored for various durations (24 h, 1 year and 2 years). For rewarming, cryovials were retrieved from LN and allowed to equilibrate for 1 hour at RT. *In vitro* germination and fertility tests were conducted with cryopreserved pollen.

Fertility assessment of cryostored pollen

For fertility studies, pollen of the dwarf hybrid cryostored for various durations (1 month, 1 year and 2 years) was utilized. Emasculation was carried out by removing from the inflorescences the portion of rachillae having male flowers once it had completely emerged out from the spathe and the female inflorescences were covered with a cotton bag (Fig. 1a) to avoid the entry of foreign pollen. A minute slit appeared in the corolla of female flowers; it took a Y shape and ivory cream color, then widened in the course of 5-6 days and fell apart at the tip of the free petals exposing the stigma once it became receptive (Fig. 1h). Cryostored pollen was dusted on the receptive stigma. Maximum receptivity of stigma was observed to be on days 1 to 3, after which it declined rapidly. For the entire inflorescence, it usually extended up to 3 to 10 days. The nut set percentage was recorded after 4-5 months. Fully matured nuts (9-10 month old) were harvested and sown in pots filled with an equal proportion of sand, soil and coir dust.

Statistical analysis: Data on pollen germination percentage and tube length were compared between treatments and their significance was derived through DMRT.

RESULTS

Pollen collection

In arecanut, selecting male flowers at the right stage was very important for pollen collection. Flowers, which were expected to open the next day, as well as those that had already opened, yielded quality pollen. Pollen

Figure 1. Viability and fertility of cryostored arecanut pollen: a) fully opened inflorescence of Hirehalli Dwarf arecanut palm; b) magnified view of opened male flowers; c) magnified view of dehisced anthers; d) collection of pollen; e) germination of fresh pollen; f) germination of desiccated pollen; g) germination of cryostored pollen; h) receptive stigma; i) nut set using pollen cryopreserved for 24 hours and (j) 2 years; k) seedling raised from nut obtained through pollination using pollen cryostored for 2 years.

yield was also affected by the drying method. Pollen quality as well as quantity improved when male flowers were dried at RT. Drying of male flowers at 30 or 32°C hindered separation of pollen and their collection from within the anthers as flowers shrunk rapidly under constant heat. Fully opened staminate flowers appeared cream in color. The quantity of pollen obtained after drying was high (5 mg of pollen from 10.4 g of dry staminate flowers) when it was taken from completely opened flowers. Pollen was scattered around the anther lobes (Fig. 1c) because of the complete breakage of sporangia.

Standardization of in vitro germination media

Desiccated arecanut pollen started swelling once it came in contact with the germination medium. After a 30 min incubation period, the pollen tubes started protruding from the pollen grains. Among the three media studied, pollen germination, as well as pollen tube length, were significantly higher on medium containing 2.5%

Table 1. Effect of sucrose concentration (%) and of arecanut genotype (Hirehalli dwarf [HD], Sumangala and their hybrid) on germination (PG, %) and pollen tube length (PTL, µm). Experiments were carried out in triplicate and data are presented with SE values. Mean values for a parameter/genotype followed by similar letters do not differ significantly according to DMRT.

	Sucrose (%)	HD	HD x Sumangala	Sumangala	Mean
PG (%)	2.5	73.9 ± 1.2	53.4 ± 1.6	47.0 ± 13.9	58.1 ± 14.0^{A}
	3.0	73.7 ± 6.1	30.5 ± 5.0	13.0 ± 4.2	39.1 ± 27.4^{B}
	4.0	70.8 ± 2.6	29.8 ± 4.5	12.9 ± 1.3	37.8 ± 25.9^{B}
	Mean	72.8 ± 3.6^{A}	37.9 ± 12.1^{B}	24.3 ± 18.5^{C}	-
PTL (µm)	2.5	235.1 ± 82.5	240.0 ± 87.5	242.4 ± 97.9	239.2 ± 87.5^{A}
	3.0	148.4 ± 54.0	147.1 ± 55.0	102.6 ± 45.5	132.7 ± 55.6^{C}
	4.0	204.2 ± 55.2	158.4 ± 71.2	96.5 ± 30.1	153.0 ± 70.2^{B}
	Mean	195.9 ± 74.2 ^{A}	180.2 ± 80.3 ^{B}	147.1 ± 93.3 ^{C}	-

sucrose irrespective of the variety tested (Table 1). Mean pollen germination and pollen tube length were 58.1% and 239.16 µm, respectively on medium containing 2.5% sucrose. Significantly higher germination (72.8%) and pollen tube length (195.9 µm) were observed in Hirehalli Dwarf compared to the hybrid or Sumangala.

Standardization of temperature for* in vitro *pollen germination

With respect to pollen germination and pollen tube length, mean values of 53.1% and 241.7 µm, respectively were observed for pollen incubated at RT (Table 2). These values were significantly higher, compared to the other two temperatures tested (30 and 32°C). Hirehalli Dwarf showed significantly higher germination (57.1%) whereas for pollen tube length, Hirehalli Dwarf (215.8 µm) and Hirehalli Dwarf x Sumangala (222.5 µm) were on par.

When Hirehalli Dwarf desiccated pollen

Table 2. Effect of incubation temperature and of arecanut genotype (Hirehalli dwarf [HD], Sumangala and their hybrid) on germination (PG, %) and pollen tube length (PTL, µm). Experiments were carried out in triplicate and data are presented with standard error values. Mean values for a parameter/genotype followed by similar letters do not differ significantly according to DMRT.

	Temperature	HD	HD x Sumangala	Sumangala	Mean
PG (%)	RT	74.9 ± 2.6	37.4 ± 2.4	47.0 ± 13.9	53.1 ± 18.3^{A}
	30°C	45.7 ± 0.4	30.9 ± 0.4	30.9 ± 0.4	29.6 ± 14.4^{B}
	32°C	50.9 ± 0.8	36.9 ± 0.5	11.9 ± 0.3	33.2 ± 17.1^{B}
	Mean	57.1 ± 13.5^{A}	35.1 ± 3.4^{B}	23.7 ± 18.8^{C}	-
PTL (µm)	RT	195.8 ± 40.7	286.9 ± 70.3	242.4 ± 97.9	241.7 ± 81.9^{A}
	30°C	208.2 ± 71.8	208.0 ± 55.0	208.0 ± 55.0	193.6 ± 68.8^{B}
	32°C	243.5 ± 65.9	172.5 ± 48.1	106.7 ± 39.9	196.9 ± 72.4^{B}
	Mean	215.8 ± 63.9 ^{A}	222.5 ± 75.4 ^{A}	192.1 ± 101.9 ^{B}	-

was stored in a bottle containing silica gel at RT, germination started declining after 3 days (Fig. 2). Germination was 74.8, 74.3 and 72.5% after 1, 2 and 3 days, respectively. Germination decreased drastically to 55.4 and 20.6% after 4 and 5 days, respectively.

Arecanut pollen cryopreservation

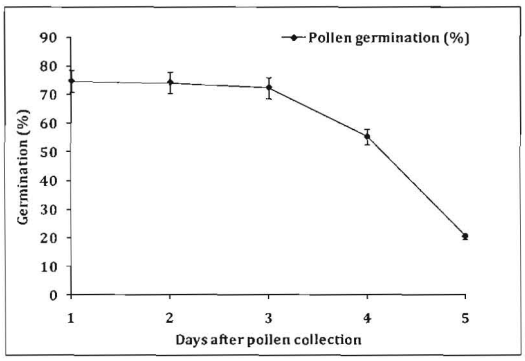

Figure 2. Effect of storage duration (days after pollen collection) on germination (%) of desiccated Hirehalli Dwarf arecanut pollen.

For the three arecanut genotypes studied, the mean germination was significantly higher in fresh pollen (77.9%) whereas it was on par for desiccated (60.9%) and cryopreserved pollen (65.9%) (Fig. 3). Hirehalli Dwarf cryopreserved pollen showed significantly higher germination (75.1%) compared to Hirehalli Dwarf x Sumangala (68.6%) and Sumangala (61.0%). However interactions between genotypes and treatments were not significant. Among the genotypes pollen tube length was significantly higher in Hirehalli Dwarf x Sumangala in fresh (317 µm), desiccated (278 µm) and cryopreserved (168 µm) pollen. In general, pollen tube length of desiccated pollen was on par with fresh pollen, but cryopreservation (139 µm) resulted in a significant reduction.

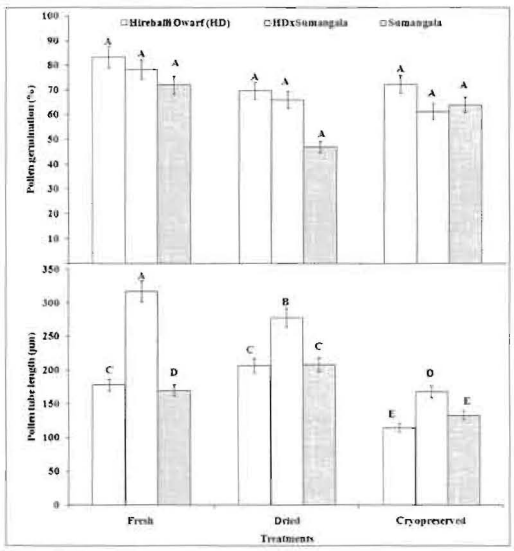

Figure 3. Germination (%) and pollen tube length (µm) of fresh, dried and cryopreserved pollen sampled from Hirehalli Dwarf (HD), Sumangala and their hybrid (HD x Sumangala) arecanut palms.

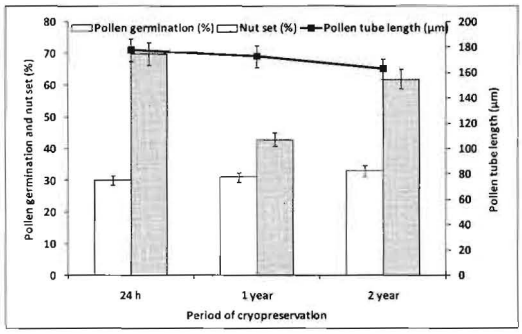

Figure 4. Effect of cryopreserved storage duration on germination (%), pollen tube length (µm) and nut set (%) of Hirehalli Dwarf x Sumangala arecanut pollen.

During the long-term cryopreserved storage experiment performed, pollen germination and pollen tube length did not change significantly and were above 30% and 163 µm, respectively, for all cryostorage periods tested (Fig. 4). Pollen cryostored for 24 hour, 1 year and 2 years showed setting of 70, 43 and 62%, respectively. The resulting nuts (Fig. 1 i & j) germinated normally upon sowing in pots and the seedlings produced were vigorous (Fig. 1k).

DISCUSSION

Pollen collection is vital both for breeding and conservation studies. Since arecanut pollen retains viability only for a few hours (25), it has to be processed immediately after opening of staminate flowers followed by dehiscence of the anthers. Moreover, male flowers mature at different periods and it takes 25 to 46 days (mean 31 days) for the whole male inflorescence to mature in a basipetal manner (20). As a result, there are few mature flowers at a time, resulting in very low pollen yield. Unopened male flowers produce immature pollen after drying. In *Papaver dubium* L., pollen maturation during the last 3 days of development occurred independently from the parent plant (9). It has been reported that pollen ages faster at high

relative humidity (RH) (35). Therefore, there is scope for further improvement of collection methods for bulk pollen yield for conservation as well as hybridization studies. Slow drying of mature male flowers at RT allowed the recovery of viable pollen in arecanut. Slow drying is usually practiced for desiccation-tolerant pollen (10), whereas fast drying of male flowers is favored for desiccation-sensitive pollen, thus extending its tolerance to freezing temperatures (3). It is important to take into account the field conditions and RH at the time of harvest as it affects pollen MC and germinability (10). Hence, it has been suggested to collect pollen during day time periods with low RH in case there is an uncertainty regarding pollen optimal MC before low temperature storage (2).

In vitro germination on artificial medium is often practiced to test pollen viability. Sugar plays an important role for artificial germination of pollen. Sugar is essential to regulate osmotic potential during pollen tube growth and it also serves as energy source (5). Furthermore, the selection of a suitable sucrose concentration is critical for pollen *in vitro* germination (6). In the present work, sucrose concentration had a strong effect on pollen germination. Maximum pollen germination and pollen tube growth were observed at low sucrose concentration (2.5%). This is in contrast another report (15) in which maximum germination of arecanut pollen was achieved with 4% sucrose. Sucrose requirement for pollen germination varies with plant species. *In vitro* germination of fresh oil palm pollen was highest on medium containing 2.5% sucrose (31). In date palm, the optimal sucrose concentration for pollen germination was 15% (19) and it was 8% for coconut (13).

Temperature has an effect on pollen *in vitro* germination and it varies with plant genera and species. In the present study, the highest temperature tested (33-34°C) was the best for pollen germination and pollen tube growth. In coconut, the optimal temperature for pollen germination is 28.0 °C (27). Studies on the effect of temperature on *in vitro* pollen germination in cotton cultivars revealed an optimum temperature of >32°C for maximum pollen germination (12). In maize, it was suggested that prolonged exposure to temperatures above 32°C reduced pollen germination of many genotypes to levels near zero (18).

In arecanut, pollen viability is 8-9 hours under normal conditions (25). Until the present work, no research had been made on the collection and storage of arecanut pollen for pollination and conservation studies. Pollen MC and storage temperature are the major factors that determine its longevity (33). Arecanut pollen is shed at the bicellular stage. Bicellular pollen can generally withstand desiccation to MCs below 11.1% DW (33). The present study revealed that arecanut pollen was tolerant to desiccation since it could be dried to 6-7% MC while maintaining high viability. However, viability in low humidity conditions (using silica gel) remained high for only 3 days, decreasing drastically to 20% after 5 days. Cryogenic storage is therefore the only option for prolonging arecanut pollen viability. Several factors such as genotype, stage of pollen, physiological status of mother-plants, cold treatment and cryopreservation protocol influence pollen post-LN survival (8). In palms, pollen cryopreservation has been successful in coconut (14), oil palm (30) and date palm (19). In the present study, germination was significantly higher in fresh pollen compared to desiccated and cryopreserved pollen. Reduction in germination of cryopreserved pollen has been reported in *Glycine* spp. (34), whereas pre-desiccated cryostored pollen of rose (26) and yam (22) did not show any decrease in germination. The speculated causes for reduced germination are differences in pollen MC and developmental stage at the time of collection but there are also unknown reasons (29). Pollen hydration status may change at anther opening, during presentation (17) or during dispersal. Accordingly pollen is classified as partially dehydrated pollen (PDP, with < 30% MC) or partially hydrated pollen (PHP, with > 30% MC) (21). Even if mature pollen grains are derived from the same mother palm or even the same flower, they may not be uniform. They may differ in MC and carbohydrate concentration depending on their position inside the loculus in the flower and on also environmental effects (23). In arecanut, there was a significant reduction in pollen tube length after cryopreservation. This is in contrast to coconut for which vigour of pollen significantly increased after cryopreservation (14). In case of maize, even though vigour, as measured by faster tube growth, has a direct influence on seedling vigour and kernel weight (24), in the present study the seedlings obtained using cryopreserved pollen retained normal vigour.

In the present study, pollen viability remained above 30% after cryostorage for 1 to

24 months, which allowed to maintain the largest possible proportion of pollen in a viable state in order to conserve the genetic composition of the original lot (32). It has been shown that seed set did not decrease when using pollen lots diluted with dead pollen, except for lots with less than 30% viable pollen (4). Hand-pollination using arecanut pollen cryostored for 1-24 months resulted in 43-72% seed setting, which was much higher compared to coconut for which 12-20% seed setting was reported (14). This shows the potential of using cryopreserved pollen for hybridization programmes and for assisted pollination for yield enhancement, which has been practiced with other horticultural crops (16, 32).

In conclusion, this is the first report of successful cryopreservation of arecanut pollen. In this work, desiccation and cryotolerance for arecanut pollen were confirmed. *In vitro* germination was optimal on medium with 2.5% sucrose. Pollen could be efficiently collected during low humidity periods of the flowering season (December to February), partially desiccated and cryostored. This will facilitate the establishment of cryobanks for long-term conservation of arecanut pollen and for its utilization in breeding programmes.

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