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Storage of cassava pollen for conservation of nuclear genetic diversity and overcoming hybridization barriers

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ABSTRACT

The objective of the storing cassava pollen is to preserve the genetic material for the future use, to maintain their germination, vigour and genetic integrity by providing the optimal conditions. Non-synchronous flowering between parental lines, monoecious and protogynous nature, poor and irregular flowering poses difficulty in cassava breeding. Staggered and multiple planting can circumvent this limitation to some extent, however it consumed a lot of time, labour and space. Availability of stored viable pollen for hybridization can be of great help as the preserved pollen facilitates the easy exchange of genetic material among the researchers as also between the nations due to the less stringent restrictions on the transfer of stored pollen. Hence, cassava pollen storage was undertaken in present studies. Freshly opened male flowers of cassava variety *Sree Padmanabha* (SP) and *Vellayani Hraswa* (VH) were collected between 9.00 AM and 10.30 AM. Viability of the pollen was tested by acetocarmine staining and *in vitro* pollen germination tests. The results showed that after 45 days of storage in liquid nitrogen, the pollen staining was 59.1% and 63.9 % while pollen germination was 49.5% and 51.0% in SP and VH, respectively in the laboratory assessment. Hand pollination in the field was done using cryopreserved pollen and the normal fruit set was observed. These findings will form the base information in helping the breeders to plan hybridization programme in cassava and in easy conservation and exchange of the elite germplasm.

Key words: *Manihot esculenta*, pollen germination, *In vitro* germination, acetocarmine test, pollen cryostorage.

INTRODUCTION

Cassava/ tapioca (*Manihot esculenta* Crantz.) is the fourth most important food crop after rice, wheat and maize. Billions of people depend on cassava as a source of calories in Africa, Asia and South America (FAO, 8). Cassava has proven to be a reliable source of food during hunger and drought periods as a result it was adopted as a popular famine reserve crop. In recent times, however, it has emerged as a food and cash crop of industrial significance (Aerni, 1). Cassava has enormous potential in India for poverty alleviation, food security and industrial uses due to its ability to grow and yield well in marginal and wasteland. Also, it offers a convenient flexibility to farmers which can be harvested as per need.

Cassava genotypes with the high tuber dry matter and starch content are suitable for industrial purpose, while cooking quality is the prime consideration for human consumption (Oliveir *et al*., 12). During the twentieth century, the remarkable increase in the productivity of many crops was due to genetic gains achieved through crop breeding. The widely used method of generating new varieties in cassava is the intraspecific hybridization between elite lines/ varieties and subsequent selection (mass selection) based on phenotype to search for superior plants within F_1s and following cloned generations (Ceballos *et al*., 2).

The crossing of elite clones in cassava has become more difficult because the flowering is genotype dependent and the environmental conditions. Some clones flower relatively early at 4 or 5 months after planting whereas others flower at 8–10 months after planting. Asynchronisation of flowering between male and female parents is also a major drawback in the cassava breeding. Besides, cassava is monoecious and protogynous. Its male flowers open 10-14 days after the female on the same inflorescence. This type of flowering behaviour in cassava slows its improvement through breeding and such circumstances, usually, make it convenient to store the pollen from male or donor parents for later pollination of the desired female parents.

Cryopreservation ensures the optimal retention of viability and genetic stability of stored tissues (Day *et al*., 4). Among the available techniques, it is a safe and cost effective method for the conservation of genetic resources in the long run. Moreover, plant propagules stored in a small volume are protected from contamination and require limited maintenance and attention (Engelmann, 6). Replenishing a small volume of LN weekly in cryo-containers is the only

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on-going maintenance operation required during cryostorage. Apart from these, further advantages of this approach are low costs of storage, minimal space and reduced labour requirement as compared to the field and even *in vitro* germplasm conservation.

In crop improvement programmes, breeders routinely store pollen in liquid nitrogen (Towill and Walters, 16). Several institutes stored the pollen for conservation of genetic resource of various plant species. In India, the Indian Institute of Horticultural Research (IIHR) Bengaluru have reported cryostorage of pollen of 600 accessions from 40 species, some samples of them have been stored for over 15 years (Ganeshan and Rajashekaran, 7) and the National Bureau of Plant Genetic Resources (NBPGR, New Delhi) have reported cryopreservation of pollen of 541 accessions belonging to different species (Mandal, 9).

Therefore, the use of stored pollen in cassava, if succeeded, could help in overcoming the difficulties in cassava breeding. Results of such studies will contribute to planned hybridization programme to generate larger progenies enabling the selection of desirable hybrids. Since the information on pollen conservation in cassava is not available, therefore the present study was undertaken to develop a cassava pollen storage protocol.

MATERIALS AND METHODS

Two popular cassava varieties for industrial and culinary purposes, *Sree Padmanabha* (SP) and *Vellayani Hraswa* (VH) were chosen for pollen storage study and profusely flowering and fruit bearing variety, *Sree Jaya* was used as female parent for pollination study. These cultivars were planted in the farm of Indian Council of Agricultural Research - Central Tuber Crop Research Institute (ICAR-CTCRI), Thiruvananthapuram, Kerala. Parent plants were maintained healthy and were free from diseases and insect pests.

The inflorescences having male buds which were ready to open next day were selected from *Sree Padmanabha* and *Vellayani Hraswa* and bagged in the afternoon hours. Next day between 09.00 AM and 10.30 AM, freshly opened male flowers of bagged inflorescences were collected, which were directly subjected to seven different storage conditions and five storage periods with three replications in each combination. For each replication, a minimum of 25 freshly opened male flowers were collected and placed in Petri dish lined with moist filter paper and brought immediately to the laboratory. The collected flowers were transferred to the respective storage conditions after placing them in the cryovials.

Cassava male flowers were subjected to seven storage conditions (room temperature, 4°C, −20°C, −80°C, −196°C, LN −20°C, LN −80°C) after placing them in cryovials. For room temperature storage (25±2°C), the vials were kept at the dark, clean and dry place (as control treatment). Storage at 4°C, −20°C and −80°C were achieved through refrigeration. By dipping in liquid nitrogen, storage of pollens at −196°C (cryostorage) was attained. In addition, for LN −20°C and LN −80°C, cryovials were dipped in liquid nitrogen for initial freezing (for about five minutes) and were placed at −20°C and −80°C refrigerators. The cassava flowers were stored for five different time intervals under different storage conditions viz., 3, 7, 14, 30 and 45 days. The samples were taken out at each time interval and tested for pollen viability using acetocarmine staining and *in vitro* germination tests with three replications in each tests.

In a test, pollen grains stained with acetocarmine were observed under microscope (Leica DM 1000, Leica Microsystems CMS GmbH, Wetzlar, Germany). Pollen grains stained in light colour, reduced and abnormal size were considered as non-viable, while those deeply stained and with larger size were classified as viable.

For *in vitro* pollen germination, pollen grains were cultured on a medium containing boric acid 100 mg/L; calcium nitrate 300 mg/L; magnesium sulphate 200 mg/L; potassium nitrate 100 mg/L; sucrose 5%. The pH was adjusted to 5.8 (Mary *et al*., 10) using sitting drop method. In this method, a drop of pollen germination medium was laid on the microscopic slide and pollen was brushed on the medium. Thereafter, the pollen was incubated for six hours at room temperature. Germinated pollen grains were counted through an optical microscope (Leica DM 1000).

Fertility/crossability of the stored pollen was tested by the controlled field pollination. Inflorescences of the desired female parents were bagged at the bud stage after removal of male flowers. Stored pollen was placed onto the receptive stigma. Pollinated flowers were immediately covered with muslin cloth bags to control cross pollination by insects and were removed after fruit set. Crosses with female parent, *Sree Jaya* were also carried out by freshly collected pollen from male parents, SP and VH. Observations were recorded on the percentage of fruit formation in all the crosses after allowing it for normal development and maturity. The tests were repeated trice (replications) and in each replication, minimum of 25 crosses were made.

The experiment was laid out in Completely Randomised Design. The analysis of pollen viability and fertility, data collected during the pollen storage

trials were subjected to two-factorial ANOVA on the effects of genotype and storage treatments by employing Minitab® software. The viability of fresh pollen of both SP and VH cultivars was compared using the two-sample t-test.

RESULTS AND DISCUSSION

The viability of fresh pollen of two cassava varieties was examined using acetocarmine staining, *in vitro* germination test, selfing and crossing with the profusely flowering female parent, *Sree Jaya*. Regardless of the varieties, differential results were obtained by different viability tests (Table 1). Comparatively higher pollen viability was observed using acetocarmine staining test; however, *in vitro* germination test and crossability tests showed a low percentage of pollen viability. Similarly, Dutta *et al*. (5) obtained the differential results from different pollen viability assays and reported that *in vitro* germination test was more reliable and overestimated results were obtained by acetocarmine tests in mango pollen viability study. Chaudhury *et al*. (3) also observed overestimation of viability with fresh and stored mango pollen. Pollen viability tests proved that fresh pollen in cassava varieties had higher viability than the stored pollen. The varietal difference for fresh pollen viability was found to be non-significant in acetocarmine test whereas a significant difference was observed for *in vitro* germination and selfing and crossing in field conditions. The maximum *in vitro* germination of fresh pollen was found in VH (67.4%) followed by SP (64.9%). Similarly, fruit set was higher in VH after selfing (61.9%) and crossing (56.2%) with female parent *Sree Jaya* as compared to pollen from SP (Table 1).

The analysis of variance (ANOVA) clearly indicated that the storage methods had the highly significant effect on pollen viability as confirmed by the acetocarmine and *in vitro* germination tests. Furthermore, the interaction between cassava cultivars and storage methods on acetocarmine and *in vitro* germination tests was statistically non-significant (Table 2 and 3).

At room temperature, pollen gave 34.2% and 33.7% staining in VH and SP, respectively during acetocarmine assay after three days of storage. After which, the staining percentage declined to zero at later stages of storage (Table 2 and Fig. 1A&B). A similar trend was also observed when pollen viability was examined using *in vitro* pollen germination; it was 16.1% and 14.3%, respectively in *Vellayani Hraswa* and *Sree Padmanabha* (Table 3 and Fig. 1C&D). Irrespective of the varieties and tests, the viability of cassava pollen was found to

Table 1: Viability of fresh cassava pollen as confirmed by acetocarmine, *in vitro* germination, selfing and crossing with female parent.

Variety	Acetocarmine (%)	Germination (%)	Selfing (%)	Crossing (%)
SP	94.3	64.9	58.7	53.5
VH	92.7	67.4	61.9	56.2
t cal	1.40	3.10*	4.35*	4.04*

*Significant at 5% level when compared with the t-table value 2.048

Table 2: Per cent stainability of stored cassava pollen at different conditions at different time intervals.

	3 days			7 days			14 days			30 days			45 days		
	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean
RT	33.7	34.2	34.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	47.6	47.8	47.7	22.4	22.8	22.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-20	83.0	81.1	82.1	76.2	79.7	78.0	72.0	69.5	70.8	35.6	41.9	38.8	25.1	24.9	25.0
-80	88.3	90.8	89.6	73.3	76.0	74.7	77.4	75.6	76.5	65.5	69.2	67.4	34.9	32.4	33.7
LN	66.4	64.9	65.7	61.8	66.1	64.0	64.2	63.1	63.7	60.7	65.1	62.9	59.1	63.9	61.5
LN -20	64.6	65.7	65.2	59.9	60.0	60.0	59.0	60.2	59.6	41.6	45.3	43.5	30.5	31.4	31.0
LN - 80	67.5	66.0	66.8	62.2	60.6	61.4	58.9	62.4	60.7	55.3	55.0	55.2	34.0	35.5	34.8
	V	T	V × T	V	T	V × T	V	T	V × T	V	T	V × T	V	T	V × T
S.E (m)	3.9	7.3	10.3	3.4	6.4	9.0	N/A	18.6	N/A	2.2	4.1	5.8	1.8	3.4	4.8
C.D. 0.05	N/A	21.2	N/A	N/A	18.6	N/A	N/A	16.9	N/A	N/A	11.9	N/A	1.8	3.4	4.8

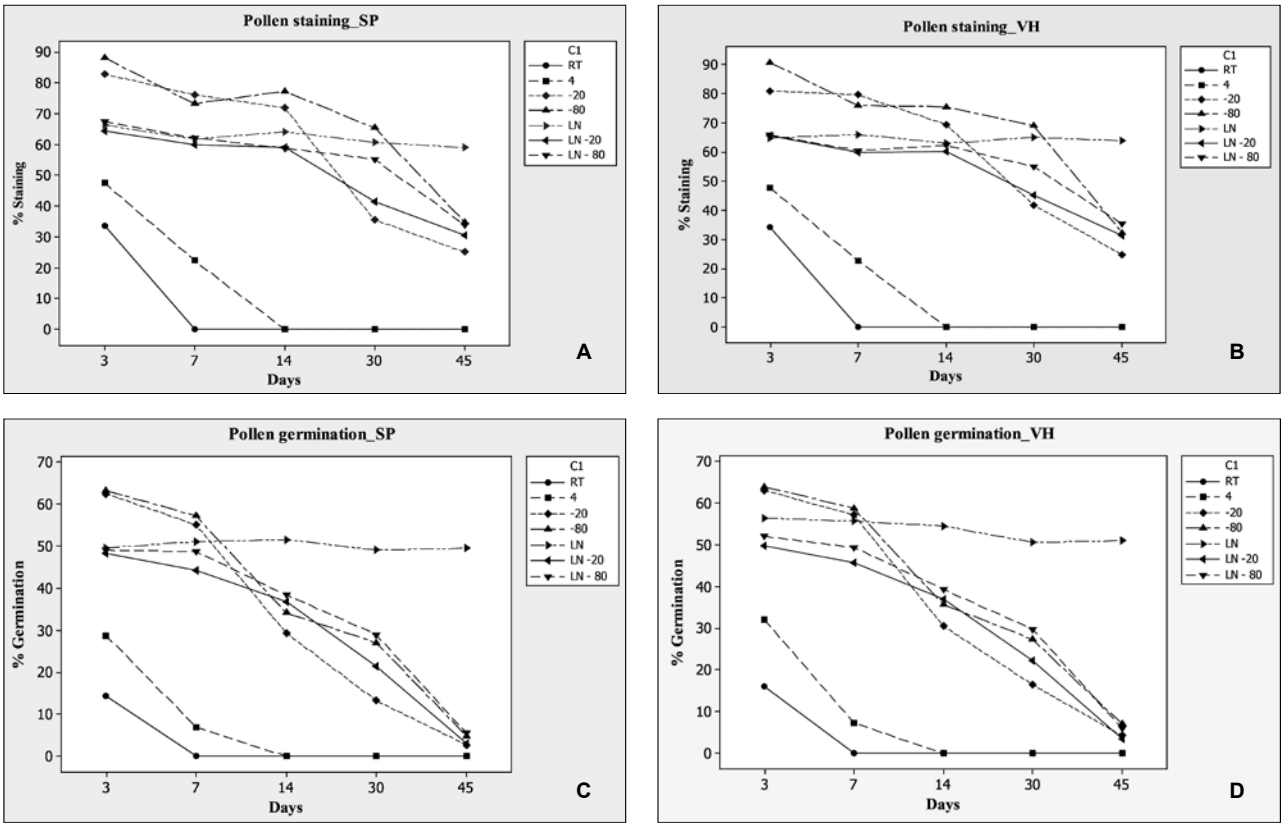

Fig. 1. Pollen viability of cassava varieties stored at different conditions evidenced by acetocarmine test (A, B) and *in vitro* germination test (C, D) at different time intervals.

be 0.0% at 7, 14, 30 and 45 days after storage at room temperature (Table 2, 3 and Fig. 1). Probably cassava pollen is highly susceptible to desiccation and there is rapid moisture loss when stored at room temperature, hence, the viability of the pollen was reduced quickly after storing at room temperature. Vieira *et al*. (17) observed 20% decrease in viability after one hour of storing fresh cassava pollen and total loss of pollen viability was recorded after 24 hours of storage at room temperature.

Pollen storage at 4°C showed non-significant differences for pollen viability among the cassava varieties on different storage durations. The viability of stored cassava pollen at 4°C was found to be decreasing significantly with the increase in storage period as confirmed by the pollen viability tests. In the variety VH, acetocarmine staining found to be 22.8% and germination was 7.2% followed by 22.4% and 6.9% in SP, which reduced to zero after 14 days of storage at 4°C in both the cultivars (Table 2, 3).

Similar to storage at room temperature and 4°C, non-significant differences for pollen viability among the cassava varieties was observed at −20°C also. The percentage of viability was high in both the varieties at different durations of storage. In SP, after 45 days of storage at −20°C, results showed a decrease to 25.1% in acetocarmine staining and 2.7% *in vitro* pollen germination. Similarly, in VH, the decrease in pollen viability after 45 days of storage at −20°C was 24.9% and 4.2%, respectively as depicted by the acetocarmine staining and *in vitro* germination test (Table 2, 3 and Fig. 1).

Pollen storage at −80°C showed similar trends to those observed in the case of storage at −20°C. The only difference noticed was that the percentage viability was higher in both varieties at all the dates of observation compared to pollen storage at −20°C. After 45 days of storage at −80°C, acetocarmine staining (34.9%) was high in SP followed by VH (32.4%). The maximum *in vitro* germination (7.0%) of stored pollen at −80°C was observed in *Vellayani Hraswa* followed by *Sree Padmanabha* (4.8%) (Fig. 1).

Storage of cassava pollens in liquid nitrogen (−196°C) showed significantly higher viability as compared to all the other storage conditions as confirmed by acetocarmine staining (Table 2 & Fig. 2a), *in vitro* pollen germination (Table 3 & Fig. 2b)

Table 3: Per cent *in vitro* germination of stored cassava pollen at different conditions at different time intervals.

	3 days			7 days			14 days			30 days			45 days		
	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean	SP	VH	Mean
RT	14.3	16.1	15.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	28.7	32.0	30.4	6.9	7.2	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-20	62.4	63.0	62.7	55.0	57.1	56.1	29.3	30.5	29.9	13.2	16.4	14.8	2.7	4.2	3.5
-80	63.2	63.9	63.6	57.1	58.7	57.9	34.1	35.6	34.9	27.0	27.3	27.2	4.8	7.0	5.9
LN	49.6	56.4	53.0	51.0	55.6	53.3	51.5	54.5	53.0	49.0	50.5	49.8	49.5	51.0	50.3
LN -20	48.3	49.8	49.1	44.2	45.7	45.0	36.7	37	36.9	21.4	22.1	21.8	3.1	3.5	3.3
LN - 80	49.0	52.0	50.5	48.6	49.3	49.0	38.4	39.3	38.9	28.9	29.7	29.3	5.5	5.9	5.7
	V	T	V × T	V	T	V × T	V	T	V × T	V	T	V × T	V	T	V × T
S.E (m)	2.9	5.4	7.7	2.5	4.7	6.6	1.8	3.3	4.6	1.4	2.6	3.6	1.3	2.4	3.3
C.D. 0.05	N/A	15.8	N/A	N/A	13.6	N/A	N/A	9.6	N/A	N/A	7.5	N/A	N/A	6.9	N/A

Table 4: Per cent fruit set of cassava pollinated from stored cassava pollen at different conditions.

	30 days			45 days		
	SP	VH	Mean	SP	VH	Mean
RT	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.0
-20	0.0	0.0	0.0	0.0	0.0	0.0
-80	12.5	12.0	12.3	6	6.5	6.3
LN	45.3	48.5	46.9	46.7	47.0	46.9
LN -20	0.0	0.0	0.0	0.0	0.0	0.0
LN - 80	11.1	13.0	12.1	6.2	7.4	6.8
	V	T	V × T	V	T	V × T
S.E (m)	1.7	3.1	4.4	1.7	3.11	4.4
C.D. 0.05	N/A	6.4	N/A	N/A	6.4	N/A

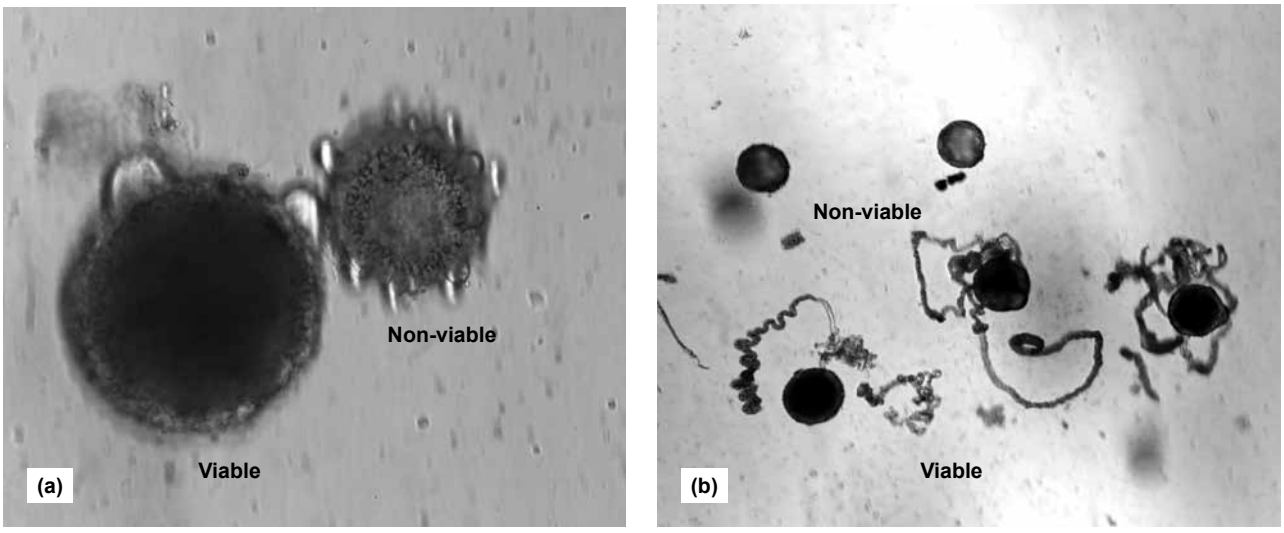

Fig. 2. Cassava pollen viability assessed by acetocarmine test and *in vitro* germination tests (a) pollen staining, (b) germinating pollen.

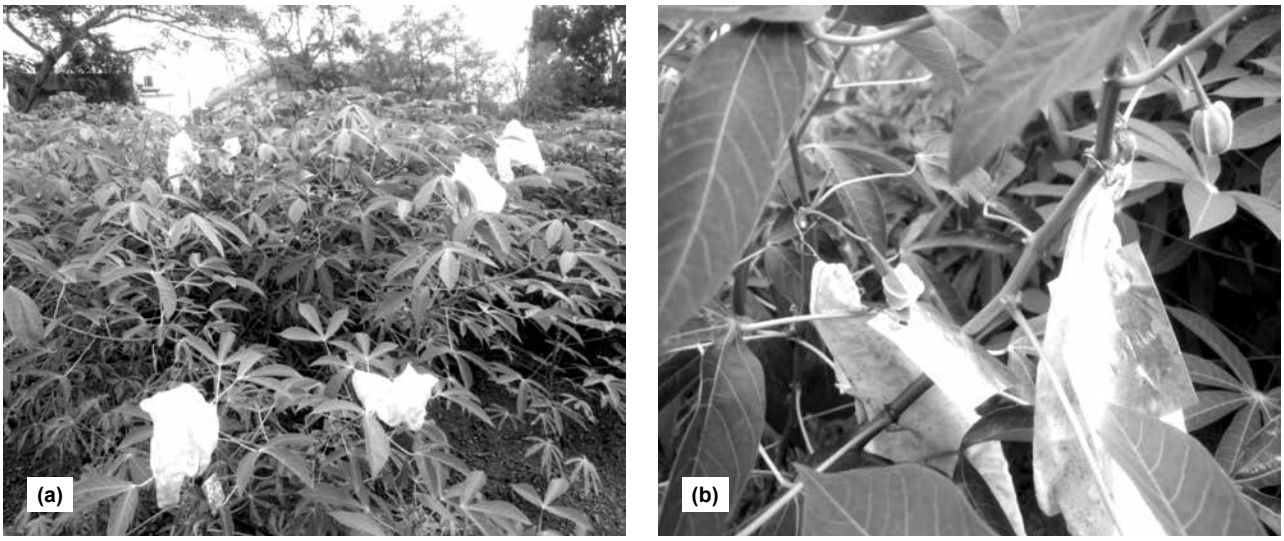

Fig. 3. (a) Pollination of female parent using stored pollen grains, (b) Fruit set by the cryo-stored pollen.

and crossability tests (Table 4 & Fig. 3). Cassava pollen stored at −196°C (liquid nitrogen) initially recorded less viability compared to the other storage conditions like −20°C and −80°C. But, the rate of reduction in pollen viability in both cassava varieties was minimal in the case of liquid nitrogen as the duration of the storage period was extended and, thus, significantly higher percentage of viability was observed at 45 days after storage (Fig. 1). Pollen grains of VH recorded maximum staining (63.9%) and *in vitro* germination (51.0%) followed by 59.1% staining and 49.5% *in vitro* germination in SP.

Under pollen storage conditions like LN −20°C and LN −80°C, the viability of pollen showed almost similar trend as observed in the case of −20°C and −80°C storage, respectively (Table 2, 3 & Fig. 1).

Crossability of the stored pollen was tested by controlled field pollinations. Profusely fruit bearing variety like *Sree Jaya* was used to test the viability of the stored cassava pollen in the field. Pollen stored for 30 days and 45 days at different conditions were used for pollination. The fruit set was observed in the pollen stored at −80°C and in liquid nitrogen (−196°C). Crossability test showed non-significant differences for pollen viability among the cassava varieties which was stored in liquid nitrogen. The maximum fruit set recorded was 47% from *Vellayani Hraswa* followed by 46.7% from *Sree Padmanabha* when 45 days old cryostored pollen was used for pollination (Table 4 & Fig. 3). When crossability test was done on *Sree Jaya* using fresh pollen from *Vellayani Hraswa* and *Sree Padmanabha*, the fruit set was 56.2% and 53.5%, respectively (Table 1). The rate of reduction in fruit set was minimal in the case of cryostored pollen compared to fresh one (Table 1 & Table 4). Mukherjee *et al*. (11) reported that the degree of successful fruit set in taro with cryostored pollen was on par with conventional fruit set. Similarly, the average fruit set in Chinese ornamental plants did not decrease when pollinated with cryopreserved pollen and in several cross combinations, it was significantly higher than the fresh one (Li *et al*., 8). Silva *et al*. (14) achieved 62.67% *in vitro* pollen germination and 70.83% seed formation in the *in vivo* pollinations with cryopreserved pollen grains of wild pineapple accessions.

The rate of reduction in the pollen viability of the cassava varieties studied was minimal in the case of cryostorage (stored at −196°C) compared to other storage conditions which make cryostorage a suitable technique for short-term as well as long-term pollen conservation in cassava. The pollen once stored at a temperature below −160°C would theoretically have an infinite period of longevity (Stanwood, 14). Dutta *et al*. (5) reported that significantly higher pollen viability was maintained due to storage at −196°C followed by −20°C and −4°C irrespective of observation dates in three Indian mango cultivars studied by different pollen viability tests such as acetocarmine, fluorescein diacetate (FDA) and *in vitro* germination. The viability of the stored pollen must be monitored before, during and after the storage. Thus, the maximum period for which pollen grains can be conserved without losing their capacity to germinate must be established (Rajasekharan *et al*., 13). Mukherjee *et al*. (11) stored the taro pollen in liquid nitrogen at different time intervals ranging from one week to two months to tackle asynchronous flowering

behaviour in taro hybridisation programme and reported that the degree of successful hybridization with cryo-stored pollen was on par with conventional hybridization.

CONCLUSION

This is the first report on the storage of cassava pollen. The results indicated that the two varieties studied did not differ significantly, but the storage methods had the highly significant effect on pollen viability as confirmed by the different viability tests. The study revealed that the cryo-storage of the cassava pollen at −196°C could be the best storage strategy for efficient conservation of nuclear genetic diversity and used for pollination in the hybridization programme. Pollen cryo-storage of differentially blooming cassava parents assures the availability of pollen during the same season, in the succeeding seasons and also in different locations. This finding would help the breeders to plan hybridization programme in cassava.

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