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Effect of low temperature regimes on storage of *Hevea* pollen

Keywords: Hevea brasiliensis, cryo preservation, pollen storage

Domesticated rubber tree, *Hevea brasiliensis* is widely cultivated for the production of natural rubber in the tropics. The tree is naturally pollinated by thrips and midges (Warmke, 1952; Rao, 1961) but hybrids are produced mainly through artificial pollinations, where synchrony of flowering and the availability of pollen is an inevitable factor. In India, main rubber flowering period is January and March. Low fruit set (Hamzah *et al.*, 2002) and non-synchrony in flowering has been reported (Varghese and Mydin, 2000) as one of the major problems in *Hevea* breeding. The necessity of pollen storage in crop breeding is well established mainly for overcoming the non-synchrony in flowering and *in vitro* preservation of germplasm (Hanna, 1990). Pollen storage has high impact on *Hevea* breeding programmes as it allows the inaccessible cross combinations. This would ultimately lead to increased number of recombinants and high variability, thereby efficiency of selection. Published reports on the storage of *Hevea* pollen are very few (Dijkmann, 1951; Hamzah and Leene, 1996), but extensive works have been done in other crops (Visser *et al.*, 1977; Rajasekharan and Ganeshan, 1994). Most of the pollen storage methods using either sub zero or very low temperatures like -196°C, facilitated increased pollen viability in many crops (Towill, 1985; Alexander and Ganeshan, 1993; Marchant *et al.*, 1993). Presence of moisture content in the pollen grains is considered as one of the critical factors, which determines the post storage viability of pollen (Hanna 1990; Niimi and Shiokawa, 1992). In *Hevea*, 7-11 percent moisture level is reported as ideal for storage upto 5 months in liquid nitrogen (LN) (Hamzah and Leene, 1996). Loss of viability is severe even for brief periods. Hence, the possibility of long-term pollen storage was tried. Objective of the present study was to evaluate and demonstrate the impact of low temperature regimes on storage of *Hevea* pollen.

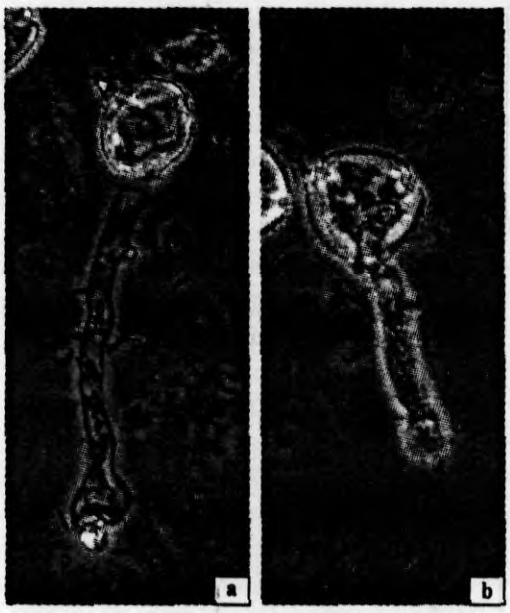
Pollen grains/anther columns from the clones RRII 105, RRII 118, RRIM 600, and PB 330 were collected. Pollen samples were taken from large number of male flowers in the flowering season. Completely ripened staminate flowers were collected between 9.00 a.m. to 11.00 a.m. every day. Flowers were brought to the laboratory and the anther columns were taken out carefully using a clean forceps and needle. Standard protocols were adopted for separation, pretreatment, germination and storage of pollen grains.

Viability of the pollen was tested by using acetocarmine stain (2.0 %) and glycerin mixture (1:1 v/v). Moisture content in the fresh pollen (20-25%) grain was adjusted gravimetrically. The moisture content in pollen samples were determined and maintained at a level of 10 percent as described by Siregar and Sweet (2000).Three replicates of each samples were made for a given time period of storage and kept in cryovials. These were stored in deep freezer (-80°C) and in LN (-196°C).Cryo vials containing pollen grains were plunged slowly into the liquid phase of LN in the cryo flasks using canisters, and maintained the level of LN through out the experiment. Germinability was tested using sucrose boric acid medium (Roberts *et al.*, 1983) with modification in the concentration of the sucrose (22 %). Stored pollen samples were recovered from deep freezer (-80°C) and liquid nitrogen (-196°C) in specific intervals. The contents of cryo tubes were put in petri dishes and the samples were thawed immediately. After 15 minutes, pollen grains were transferred in to clean microscopic slides. Viability scoring was done using the hanging drop techniques (Shivanna and Rangaswamy,

1992). Pollen grains with length of tubes over and above the diameter of the pollen were considered as viable.

Observations were recorded from replicated samples. After germination test, stored pollen samples were disbursed into same germination medium. Small portions of pollen dispersions (10 µl) were checked for the pollen load. Only female flowers which are likely to open in same day of pollination were retained and used for pollination. Drops of pollen dispersions were applied onto the stigmatic surface of female flowers in the emasculated inflorescence using polyethylene transfer pipette fitted with 100 ml micropipette tips. The pollinated female flowers were sealed with cotton wool and latex to avoid contamination. Respective fresh pollen was used to pollinate female flowers in the same manner served as control. Counting of fruit set was done as reported earlier (Chandrasekhar *et al.*, 2004) and the fruits were labeled and left for further observations. From the data on number of crosses effected with preserved and fresh pollen and number of fruit set obtained after field pollination, percentage of fruit set was determined. Student's t test to determine the significance of the difference between control and the treatment was done.

Comparative morphology of the germinated pollen grains of -80°C stored pollen grains is shown in the Fig. 1. Germinated pollen grains did not show any significant variation in the morphology of the stored pollen (Fig. 1a) compared to that of fresh control pollen with moisture content of 20 percent (Fig. 1b). Pollen samples of four *Hevea* clones *viz.*, RRII 105, PB 330, RRII 118 and RRIM 600 were taken for the storage studies in liquid nitrogen. Kinetics of rate of *in vitro* germination obtained after storage of pollen in -196°C for different durations is shown in the Table 1. The clone RRIM 600 showed higher average germinability until 8 weeks. Germinability ranged from 10.1 to 14.7 when compared to the control with an average of 18.1 percent germination. Germinability of RRIM 600 pollen did not reduce much over time. Significantly low viability of one percent was noted in RRII 105, 8 weeks after storage in liquid nitrogen. However, the pollen sustained viability until 4th week invariably in all four clones. Thereafter sudden loss of viability of stored pollen was observed, as reflected in the percentage of germination. In PB 330, the pollen viability was reduced from 21 percent to a level of 3 percent in the 8th week after storage. Whereas, in clone RRII 118, germinability of pollen did not sustain even after 2 weeks of preservation, with 10 per cent germination. Comparatively the highest viability was recorded for pollen grains of RRIM 600 after 8 weeks of storage in -196°C when compared to the other pollen samples. Data on percentage germinability of all four clones studied demonstrate that storage of pollen is possible up to 4th week in present storage conditions.



Phase contrast microscopic photographs showing morphology of germinated pollen grains of clone RRII 105 stored in -80°C for 12 months (a); and that of fresh pollen (b) as control.

Fig. 1. ***In vitro*** **germination of** ***Hevea*** **pollen**

Table 1. Kinetics of in vitro pollen germination after storage of pollen in -196°C

Clones	Percentage of pollen germination			
	Control (Mean ± SD)	2 weeks (Mean ± SD)	4 weeks (Mean ± SD)	8 weeks (Mean ± SD)
RRII 105	26.86 ± 6.8	21.04 ± 4.69	18.22 ± 4.53	1.05* ± 2.49
PB 330	24.48 ± 5.91	21.04 ± 4.69	20.98 ± 6.21	3.33* ± 3.38
RRII 118	22.36 ± 9.47	10.00 ± 6.17	8.60 ± 5.04	1.14* ± 2.37
RRIM 600	18.14 ± 4.00	14.77 ± 4.93	11.7 ± 5.63	10.17 ± 2.22

** Not significant*

Pollen grains of different Hevea clones stored in LN and the samples were recovered germinated in sucrose-boric acid medium and the percentage of germination was scor Values are mean ± SD, $p<0.05$ compared to control values.

Pollen grains of *Hevea* clones viz. RRII 105, PB 330, RRII 118 and RRIM 600 were also stored in -80°C and the data on pollen germinability before and after storage at -80°C is shown in the Table 2. Pollen viability of 25 per cent was retained by the RRII 105 pollen grains up to 10th month and at 12th month viability declined to 18 per cent. There was no significant pollen germination in the samples stored beyond 12 months. Pollen grains of clone RRII 118 showed appreciable pollen viability, only up to one month (19.0%) and thereafter showed a decline to 4.1 percent; whereas RRIM 600 recorded a pollen germination of 30.8 % upto 3 month of storage in -80°C.

The siring ability of stored pollen was tested by pollinating the female flowers of clones RRII 105 and

Table 2. Kinetics of in vitro pollen germination after storage in -80°C

Clones	Duration of storage (months)								
	Control	1	2	3	6	8	10	12	13
RRII 105	35.9±6.1	31.3±6.3	30.3±5.4	29.9±5.4	27.6±5.2	27.8±5.3	25.6±4.7	18.5±3.5	4.5±3.0
PB 330	30.0±6.4	20.5±3.9	3.5±2.8	--	--	--	--	--	--
RRII 118	24.9±4.1	19.0±4.4	4.2*±3.7	4.1*±2.5	--	--	--	--	--
RRII 600	34.6±4.0	34.2±3.8	28.8±4.3	30.8±4.7	9.5±2.3	--	--	--	--

**Not significant*

Pollen grains of different Hevea clones stored after reducing the moisture content to 10 percent in –80°C. The samples were recovered and germinated in sucrose-boric acid medium and the percentage of germination was scored. Values are mean ± SD, $p<0.05$ compared to control values.

PB 330. Fruit-set obtained after 30 days and 120 days was recorded and compared with that of control, where the respective fresh pollen was used and percentage fruit set was worked out. Table 3 shows the details of field pollinations done using stored pollen grains in -196 °C and in -80 °C. LN stored pollen grains of RRII 105 for a period up to four weeks which showed an average of 18.2 percent germinability in the *in vitro* conditions were used to fertilize the female flowers of PB 330. The result shows that, five out of the 100 female flowers fertilized, set fruits and was retained in the tree with an initial fruit set percentage of 5 per cent; and only two per cent of fruits were finally retained 120 days after pollination. Those flowers fertilized with fresh pollen of RRII 105 also set five per cent fruits. Subsequently, at maturity two fruits could be harvested from the treatment and 2.5 per cent fruit retention were obtained from the control where fresh pollen was used. Thus the overall percentage from the female flowers pollinated with pollen grains, stored at -196 °C in liquid nitrogen for four weeks is two per cent when compared to fruit set percentage of flowers pollinated with fresh pollen. Similarly pollen grains of the clones RRII 118 stored in the liquid nitrogen for two weeks were used to fertilize 150 female flowers of PB 330. Initial fruit set was 10 per cent when compared to that of control, where 12 per cent of the female flowers set fruits. However, the number of fruits retained after four months was two per cent for both treatment and control. The fertilizing capacity of pollen from RRIM 600 tested in the female flowers of PB 330 showed the initial fruit set of five percentage, compared to control with six per cent. From three percent of fruits were obtained after four months. In the case of PB 330 pollen, the corresponding female parent RRII 105 recorded a fruit set of five per cent initial success and none of these fruits were retained up to maturity; but those flowers pollinated with fresh pollen (control) retained two percentage fruit set until maturity. It is clear that genetic make up of the pollen also might have played a role in the longevity of viability retention. Level of moisture content is reported to have high impact in the pollen storage; as reported in other crops (Hanna, 1990; Hamzah and Leene, 1996).

In order to confirm the siring ability of pollen of clone RRII 105 stored up to 12 months period in -80°C was taken for the field fertilization in the female parent PB 330. From the 100 pollinations attempted with stored pollen in -80°C, 5 per cent fruits were formed compared to those female flowers pollinated with fresh pollen grain with 10 per cent success one month after pollination. However, final fruit set percentage of control and treatments was six per cent and two per cent, respectively. Percentage of success reported here is almost nearer to the reported values, where conventional methods were employed (Morris, 1929; Ross, 1960; Attanayake, *et al.*, 1984; Mydin *et al.*, 1990; Hamzah *et al.*, 2002). However, reports on cryostored *Hevea* pollen are rare except the few (Hamzah and Leene, 1996).

Table 3. Details of artificial pollination using preserved pollen grains of *Hevea*

Parentage			Period of storage (weeks)	No. of pollinations attempted	Fruit-set 30 days after pollination		Fruit-set 120 days after pollination	
Female		Male			Number	Percentage	Number	Percentage
PB 330	x	RRII 105	4	100	5.0 (5.0)	5.0 (6.0)	2.0 (2.5)	2.0 (2.5)
RRII 105	x	PB 330	4	100	5.0 (7.0)	5.0 (7.0)	0.0 (2.0)	0.0 (2.0)
PB 330	x	RRII 118	2	150	16.0 (18.0)	10.6 (12.0)	2.0 (3.0)	2.0 (2.0)
PB 330	x	RRIM 600	2	100	5.0 (6.0)	5.0 (6.0)	3.0 (2.0)	3.0 (2.0)
PB 330	x	RRII 105*	58	100	5.0 (10.0)	5.0 (10.0)	2.0 (6.0)	2.0 (6.0)

Pollen grains of different clones stored in -196°C and -80°C were recovered assessed the germinability and used for pollination and fruit-set observed. Figures inside the brackets show the control values*

Pollen from four *Hevea* clones, RRII 105, RRII 118, RRIM 600 and PB 330, which showed non-synchrony in flowering, was used to evaluate two low temperature storage methods in -80°C and -196°C. Field pollinations using stored pollen and fruit set after pollination was studied. Pollen from RRII 105, stored at -80°C retained viability up to one year. Pollen from RRII 105, PB 330 and RRIM 600 stored at -196°C in LN maintained viability only up to one month of storage. Thereafter sharp decline in the viability was observed when compared to the fresh pollen as control. Two per cent fruit set was recorded when 4 week stored pollen grains of RRII 105 in LN (18.2 per cent germinability) was used to fertilize the female parent PB 330. RRII 105 pollen grains stored at -80°C for a period of one year could produce two per cent fruit set in PB 330. Germination of pollen grains showed wide variation among different clones. Results suggest that storage in -196°C one month and storage in -80°C for one year are effective methods for pollen storage in *Hevea* clones. Pollen storage in -80°C is more easy, practical and inexpensive. This could be useful in keeping the pollen readily available for crossing and establishment of haploid gene pool or a pollen repository for *Hevea* clones.

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