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Pollen cryo-preservation for hybrid seed production in hot pepper

RAKESH C. MATHAD[1*], S. B. PATIL[1], G. Y. LOKESH[1] AND B. A. SAVEGOWDA[1]

[1]Seed Unit, University of Agricultural Sciences, Raichur-584102
[2]Dept. of Seed Science and Technology, University of Agricultural Sciences, Bheemarayangudi
*rakeshcmrnr@gmail.com

ABSTRACT Hot Pepper is one of the important vegetable crops of Asia. In hot pepper, seed production is mainly done using cytoplasm male sterile lines. For seed production, the male line is grown in a centralized facility and male buds are distributed to growers for pollen extraction and crossing of female buds. An effort was made to preserve pollen from these male buds under refrigeration and cryo-preservation, which can be used to directly supply pollen, instead of buds, to longer distances. The pollen from these male lines were harvested and stored in normal room temperature (25°C-Control), in deep refrigeration (-20°C) and in liquid Nitrogen (-196°C). The pollen lost viability after 3 days and failed to produce seeds when stored under room temperature and after 6 days in deep refrigeration. In case of cryo-preservation, the pollen could be stored up to 47 days and produced seeds up to 48 days. The pollen cryo-preservation helps in reducing cost of seed production and ensures germplasm security.

Keywords: Pollen cryo-preservation, hot pepper, pollen viability

India is having the unique distinction of being a major seed production hub among the Asian countries due to its diversified agro climatic regions. Hence most of the tropical vegetable seeds are produced in India with the exception of some *brassica* pecies which need temperate conditions. The asian type hot pepper is an important vegetable for which the commercial hybrid seed production is going on in a very large area. The seed production is carried out by farmers and they gain high returns per unit area. The seed production of hot pepper is a skilled activity which involves emasculation and pollination. The usual practice is to grow cytoplasmic male sterile lines and fertile male plants separately and pollen from fertile male plants is used to pollinate the male sterile lines. The entire process is very costly. Sometimes crop failure may jeopardize the entire seed production programme. In an experiment conducted at UAS, Raichur, an effort was made to store pollen of hot pepper in liquid nitrogen which can be used for pollination at various sowing windows without growing male fertile lines separately. The research on storing of pollen in extreme low temperature is almost a century old. The possibility of preserving pollen to ensure fertility of crop species in breeding programmes is proved earlier. The use of pollen cryo-preservation for hybrid seed production is having more potential in high value crops of *solanaceae* (hot pepper, tomato, capsicum and egg plant) where hand pollination is the main method to produce hybrid seed. Earlier studies in hot pepper reported that hot pepper pollen can be stored in liquid nitrogen [1,2]. One important study reported that the pollen of capsicum can be stored for more than 42 months without any effect on the pollen viability and fertility [3]. In other crops, pollen storage facilitates cropbreeding, genetic conservation and artificial pollination [4]. The Cryo-preservation technique has been found viable for long term storage of pollen in several crops]. The pollen stored at low temperature has been reported to show better germination capacity [5].

MATERIALS AND METHODS

Collection of flower buds, pollen extraction and storage

For the study, flower buds from fertile plants were collected from the crop grown in the previous season. The flower buds were plucked from the plants and stored in wet cotton cloth. The flower buds were then exposed to sunlight to induce anther dehiscence. The pollen was collected in a container made of china clay and then transferred to various containers based on the type of storage. The pollen were stored in capped vials for normal storage (under cool conditions under room temperature) and for deep refrigeration (at -20°C). The semen straws were used for storing pollen under liquid nitrogen. These straws were normally used for artificial insemination in department of animal husbandry. The IBP cryo-cans were used for cryopreservation. These cans are having capacity of 3.9 L with evaporation rate of 1.8L for 14 days. The technical specifications of the cryo-cans are given in

*Corresponding Author

Table-1. The commercial grade liquid nitrogen was obtained from Karnataka Milk Federation. The cryo-cans were monitored regularly as per the earlier methods [6] and filled with fresh liquid nitrogen after every fortnight and care was taken to maintain at least 50% liquid nitrogen at all times.

Table 1. Technical specifications of cryo-cans of 3.9 L capacity used for pollen preservation

Sl No	Particulars	Unit	Technical details
1	Capacity LN2	Litres	3.9
2	Empty weight	Kgs	3.3
3	Full weight	Kgs	6.4
4	Neck diameter	mm	51
5	Outer diameter	mm	220
6	Total height	mm	500
7	Static Evaporation Loss	L/day	0.15
8	Static Holding Time	Days	29
9	Canister OD	mm	38
10	Canister height	mm	120
11	No of canisters	No.	6

Stage of pollen development for pollen collection

To collect pollen grains for long term storage, flower buds were selected when they were minimum 4-6.5 mm in length (bud stage-III) as described by Erickson and Markhart [7].

Study of pollen viability and germination

For pollen viability study, 2% acetocarmine was prepared by dissolving 2 gm carmine powder in 55 ml of distilled water and 45 ml of acetic acid. This mixture was boiled gently for 5 minutes and filtered with No-75 Whatman filter paper after cooling. For studying pollen viability, the pollen grains were placed on a cavity slide containing 2 drops of 2% acetocarmine. These were mixed using a clean needle and examined under microscope for staining pattern. The staining patterns were classified as dark, light and un-stained. The pollen grains which were stained dark and light were taken as viable. Pollen grains were observed randomly from average of five microscopic fields and counts of viable and non-viable pollen were recorded. The pollen germination has been also studied previously using hanging drop technique [8].

Determination of pollen moisture content (PMC)

If the moisture content of pollen is more than 30%, it affects its quality and has an impact on its viability [9]. The ideal PMC for storage and subsequent hybridization should be not more than 6% where it is free from fermentation and rapid deterioration [10]. PMC by Karl Fischer titration was performed according to the method proposed by Gergen *et al.* [11], using an automatic METROHM Titrino 785 titrator (Herisau, Switzerland). Titrations were performed using apura Titrant 5 (Merck) as the titrant and a mixture of methanol p.a. (Merck) and n-octanol alcohol p.a. (Vetec) 1:1 (v/v) as solvents. The polarization stream for potentiometric determination of reaction endpoint was 10 mA and titration end point voltage was 100 mV. The titration was performed using a jacketed reaction vessel with temperature adjusted and kept constant by a thermostatically heated water bath.

PMC by Gravimetric method was also evaluated whrein 2 g of pollen sample were transferred to tared aluminum capsules, weighed and placed in a vacuum oven at a temperature of 70° C for 8-10 h, until constant weight was attained [12].

Study of fruit and seed quality parameters

The pollen, after storage under various conditions, was used to pollinate the flower buds of male sterile plants. Each day, ten previously tagged flower buds were pollinated. For ease in identification, various colour tags were used. The seeds were extracted from tagged fruits at 95 % colour development or Breaker Red stage as per the subjective colour scale developed by Krajayklang *et al.* [13].

Collection of data and analysis

The data was collected every day for the first seven days and later, once in five days up to 47th day. The seed quality parameters such as fruit set (%), average seed set per fruit (No.) and seed germination (%) were recorded. The data was recorded in three replications and the experimental results were analyzed statically using randomized block design by adopting the analysis of variance technique as described by Panse and Sukhatme [14] .

RESULTS

The results of the experiment are presented in Table 2. The results indicate that the pollen germination was better for 3 days under ambient storage, for 6 days when stored under deep refrigeration and for 47 days when stored in liquid nitrogen. On the first day of pollen storage at room temperature, the pollen germination was 85% which reduced to 17% on third and last day. Similarly, pollen stored in a deep freezer produced pollen germination of 85 % on the first day. The pollen remained viable for six days and recorded 11% on the last day. The pollen stored in liquid nitrogen remained viable for 47 days with 85 % pollen germination on the first day, improved to 86% on the second day but gradually dropped to 7% on last day. Though the pollen germination was observed in all storage methods, a decreasing trend was recorded (Fig. 1).

The fruit set % was better when pollen was stored for 3 days (99, 88 and 56%) under ambient room temperature. In case of deep refrigeration, fruit set could be obtained up to 7 days (99 to 24%). Better fruit set % was obtained up to 47 days, when pollen which was stored in liquid nitrogen was used, though with decreasing trend (98 to 13%) (Fig. 2).

The results also showed that higher seed set per fruit was obtained when pollen which was stored in liquid nitrogen up to 47 days was used (168 seeds per fruit on first day to 5 seeds per fruit on 47[th] day). Very low seed setting was observed (only for 3 to 6 days) in case of pollen stored under ambient room temperature and deep refrigeration (Fig. 3) because the pollen viability lasted only for 3 and 6 days respectively. Though the average seed setting is better in cryo-stored pollen an economically viable seed setting of at least 20 seeds per fruit can be achieved upto 37 days. The other storage methods failed to achieve economic seed setting beyond six days. The pollination of hot pepper is usually done for 30 to 35 days to get good average seed setting. Thus cryopreservation can be used for pollination and hybrid seed production without growing male inbred lines afresh.

The seed germination showed no significant reduction with respect to storage methods and ranged between 64-84 % (Fig. 4). This showed that all the pollen storage methods produced good seed germination even with lowest fruit and seed setting.

DISCUSSION

The decreasing trend in the pollen germination in all pollen storage methods indicate that the temperatures above 0°C result in decline in the pollen viability. These results prove the earlier findings where moisture content of the pollen and humidity of the storage

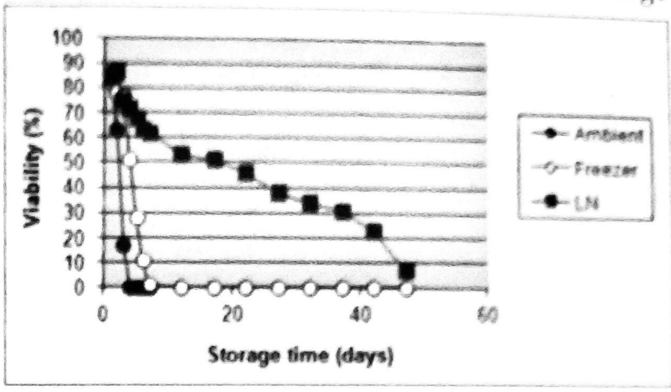

Fig. 1. Effect of storage time on pollen germination

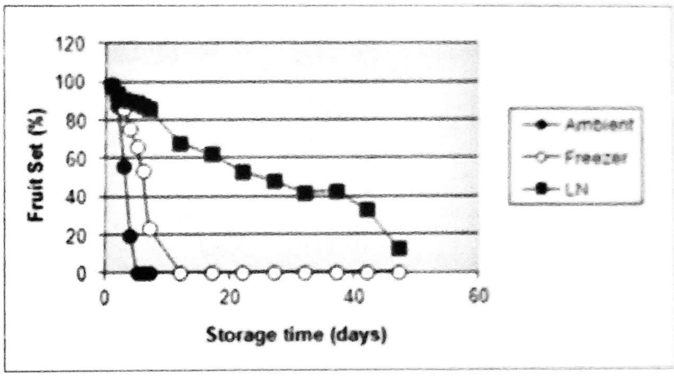

Fig. 2. Effect of storage time on fruit set

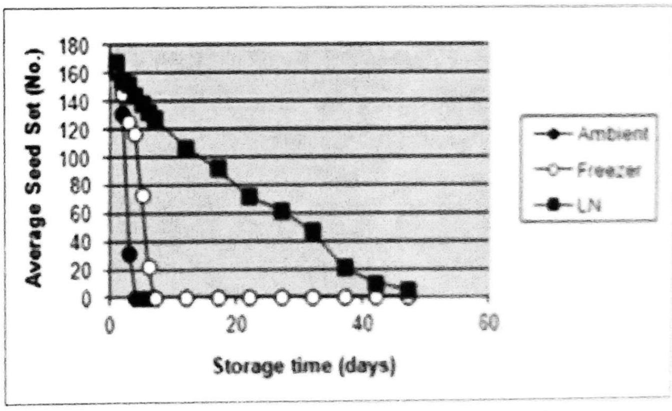

Fig. 3. Effect of storage time on seed set

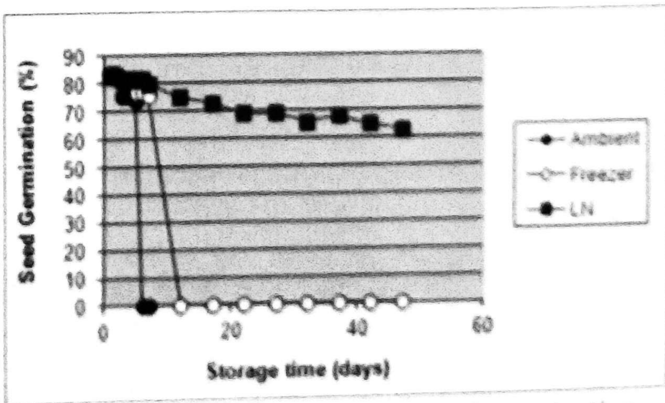

Fig. 4. Effect of storage time on seed germination

Table 2: Influence of cryo-preservation on pollen and seed quality parameters

Storage Days (T)	Pollen Germination %				Fruit Set %				Average Seed Set- No.				Seed Germination %			
	C-1	C-2	C-3	m	C-1	C-2	C-3	m	C-1	C-2	C-3	m	C-1	C-2	C-3	m
T1- 1 DAS	85	85	85	85	99	99	98	99	161	166	168	165	84	84	83	84
T2- 2 DAS	63	78	86	76	88	92	94	91	132	146	155	144	83	84	83	83
T3- 3 DAS	17	77	75	56	56	86	91	78	32	125	153	103	76	82	82	80
T4- 4 DAS	0	51	72	41	20	76	90	62	0	117	145	87	76	81	82	80
T5- 5 DAS	0	28	68	32	3	66	89	53	0	73	139	71	74	77	82	78
T6- 6 DAS	0	11	63	25	0	54	88	47	0	23	133	52	0	77	82	53
T7- 7 DAS	0	1	62	21	0	24	86	37	0	0	128	43	0	76	80	52
T8- 12 DAS	0	0	58	18	0	0	68	23	0	0	107	36	0	0	75	25
T9- 17 DAS	0	0	51	17	0	0	62	21	0	0	92	31	0	0	73	24
T10- 22 DAS	0	0	46	15	0	0	53	18	0	0	72	24	0	0	69	23
T11- 27 DAS	0	0	38	13	0	0	48	16	0	0	62	21	0	0	69	23
T12- 32 DAS	0	0	34	11	0	0	42	14	0	0	47	16	0	0	66	22
T13- 37 DAS	0	0	31	10	0	0	43	14	0	0	21	7	0	0	68	23
T14- 42 DAS	0	0	23	8	0	0	33	11	0	0	10	3	0	0	65	22
T15- 47 DAS	0	0	7	2	0	0	13	4	0	0	5	2	0	0	63	21
Mean	11	22	53	29	18	33	67	39	22	43	96	54	26	37	75	46

	Pollen Germination S.Em ±	Pollen Germination CD at 5 %	Fruit Set S.Em ±	Fruit Set CD at 5 %	Seed Set S.Em ±	Seed Set CD at 5 %	Seed Germination S.Em ±	Seed Germination CD at 5 %
T	0.74	1.81	0.56	1.81	0.65	1.81	0.33	1.81
C	1.65	3.1	1.25	3.1	1.46	3.1	0.73	3.1
T X C	2.86	1.6	2.17	1.6	2.53	1.6	1.27	1.6

DAS Days after storage
C-1 Pollen stored in ambient room temperature
C-2 Pollen Stored in deep freezer (-20 °C)
C-3 Pollen stored in liquid nitrogen (-196 °C)

atmosphere played important role in reduction of pollen viability [15,16]. However, the gradual loss of pollen viability may be due to drying and thawing effect. Normal seed setting using cryo preserved pollen has also been reported by Crisp and Grout [17]. The pollen germination of hot pepper after storing in liquid nitrogen showed that pollen can tolerate extreme ultra low temperature and still effect normal fertilization. This is due to very low biological activity at these temperatures for pollen deterioration [18]. Also the pollen deep freezing or cryo-preservation does not produce any phenotypic abnormalities which might influence the seed setting. This finding is in line with observations recorded in pollen storage studies of maize [19].

The better fruit set with cryo-preservation is also in conformation with earlier reports of higher fruit and seed set % by using cryo stored pollen [20]. This also proves the fact that normal fertilization and fruit set is possible when pollen survival in liquid nitrogen is at least 10% [21]. This can be correlated in the present study as well, where the cryo-preservation of pollen produced at least 10% pollen germination up to 37 days. The decreasing trend with reference to fruit set and seed setting is again attributed to the moisture content of the pollen before storage which varies with pollen cell type [22]. For better longevity of pollen it is necessary to lyophilize pollen before storage [19, 23]. The pollen longevity has been reported to be extended by using lower temperatures with lower moisture content in pollen of *Mangifera* [24].

Despite the decreasing trend in pollen viability, seed set occurred to a certain extent. This observation deviates from earlier results where fertilization failed even after pollen germinated *in-vitro* [25, 26]. The higher seed set using cryo stored pollen could be due to sufficient quantity of viable pollen to achieve economic seed setting. This finding is in line with findings of Choudhury *et al.* [24]. The cryo-preservation of pollen along with other methods of storage resulted in normal seed germination but there are differences when fresh pollen and stored pollen are used. This finding deviates from the earlier observation in *Prunus spp.*, where cryo-stored pollen produced better seed germination than the fresh pollen [27]. The current study showed there is no adverse effect of cryo-preservation of pollen on seed germination which is not in agreement with and contradicts the findings of Crisp and Grout [17] in *Broc coli*, where seeds produced with cryo stored pollen lost germination capacity. Also there was no slow germination of seeds observed in the present study, as described by them.

The current study therefore proves that the pollen stored under ultra low temperatures can be used for pollination without affecting the seed germination. This will help in reducing the cost of seed production and ensure germplasm security. However the storage storability of hot pepper seeds produced from cryo preserved pollen should be analyzed. A more specific protocol for lyophilizing pollen and use of cryo protectants for cryo storage are the future lines of study.

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