

## Freeze preservation of gladiolus pollen \*

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### Abstract

Viability and fertility profiles of cryopreserved gladiolus pollen from 7 cultivars have shown that it is possible to use cryogenic methods for conservation and management of the haploid gene pool in this species. There was no decline in pollen viability (*in vitro*) levels after 1 and 10 years of cryogenic storage. Field pollinations with cryogenic stored pollen induced capsule and seed set in varying capacities. Long-term cryogenic storage of gladiolus pollen could enhance breeding efficiency through better management of the haploid gene pool resources. Pollen parents could be made available throughout the breeding programme, ensuring guaranteed supply at the time of peak stigma receptivity. A 'pollen cryobank' facility established for this species would increase genetic diversity conservation at the haploid stage.

### Introduction

Gladiolus is one of the most important bulbous ornamental crops which is grown in many parts of the world as either cut flower or for its garden display. Modern cultivars have been bred by introgression of genetic traits derived from several species and hence it becomes difficult to distinctly assign cultivars to different species (Misra & Singh, 1989). Prolonging pollen viability through pollen storage is reported in many crop species as a means for overcoming asynchrony in flowering, scheduling hybrid seed production and haploid gene pool conservation. However, published reports on this aspect are restricted to a few ornamental crops. Hughes & Lee (1991) successfully preserved *Clianthus* pollen at - 180° C. Marchant et al. (1993) cryopreserved pollen of two English rose cultivars for 8 weeks. Among bulbous ornamentals, long-term pollen storage of *Narcissus* in liquid nitrogen is reported by Bowes (1990). In gladiolus, pollen storage for a duration of 730 days at - 40° C has been reported by Koopowitz et al. (1984) for 5 species.

The present study demonstrates the feasibility of cryogenic storage of gladiolus pollen and establishes

the viability and fertility status after a duration of one and ten years.

### Materials and methods

The plant material was raised at the experimental farm of the Indian Institute of Horticultural Research (IIHR) Hessaraghatta, Bangalore, India. The following gladiolus cultivars were selected for this study:

'Apsara', 'Meera', 'Nazrana', 'Poonam' and 'Sapna' which are hybrid varieties developed and released by IIHR (Raghava et al., 1981; Negi et al., 1982). They were promising in terms of flower colour, as cut flowers with a long vase life and as excellent plant material for garden display. 'Poonam' is a prolific corm multiplier and tolerant to *Fusarium* wilt disease and 'Sapna', 'Nazrana' are also established as excellent multipliers. 'Shobha' is an induced mutant from 'Wild Rose', through 1 kR gamma irradiation. 'Jowagenaar' is an exotic introduction suitable for cut flower and garden decoration. A promising hybrid, 'Dhiraj', was used as a seed parent for pollinations with 10 year cryogenic stored 'Jowagenaar' pollen.

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Pollen samples were collected, as given in the schedule below, for different experiments:

- |    |  |   |
|----|--|---|
| 1. | Apsara<br>Meera<br>Poonam<br>Sapna<br>Shobha | October 1991 cryopreservation feasibility   |
| 2. | Apsara<br>Meera<br>Nazrana<br>Jowagenaar     | October 1991 cryopreserved for 502 days<br>October 1991 cryopreserved for 476 days<br>October 1991 cryopreserved for 503 days<br>March 1983 cryopreserved for 3668 days |

#### *Cryopreservation feasibility experiment*

This experiment was carried out to ascertain whether *gladiolus* pollen could withstand cryogenic freezing, using 5 selected cultivars. This had to be carried out (i) as a precautionary step before thawing out pollen samples cryopreserved for the longest duration (Jowagenaar), (ii) for further continuing freeze preservation of pollen samples upto the next ensuing season.

The basic assumption (null hypothesis) made was that, cultivar specific responses to cryogenic freezing may not be significant. Besides this, the occurrence of asynchrony in flowering restricted inclusion of more cultivars under feasibility tests.

#### *Pollen collection and processing for storage*

The flowers were tied with thread at the bud stage in order to prevent contamination by stray pollen and to obtain pure samples. On the day of collection, the flowers were harvested and brought to the laboratory. Petals were carefully separated and pollen grains were extracted by scrapping the mature anthers which were about to dehisce, with a blunt needle, passing transversely along the lobe of the anther. Care was taken not to scrape off the tapetal tissue, which could contaminate the pure pollen. After initial viability assessment, bulked pollen samples were transferred to empty gelatin capsules, packed in laminated poly aluminium pouches, sealed airtight, stacked and lowered gradually into cannisters of a liquid nitrogen cryobiological system (Mach SM-43, MVE USA). Pollen samples were cryopreserved for different durations as per the experimental design followed for different cultivars.

#### *Viability assessment*

Viability of fresh and cryopreserved samples were indexed as a function of pollen germinability *in vit-*

*ro*, by the improved cellophane method. Fresh *gladiolus* pollen germinated without pre-hydration by this technique (Alexander & Ganeshan, 1989). The method consisted of stapling together on one edge seven 40 mm square sheets of Whatman No. 1 filter paper in the form of 'booklets'. Fifteen mm square non-water proof cellophane (0.01 mm thick) was inserted between the top and second sheet of each booklet and placed in the nutrient medium. The nutrient medium consisted of 15% sucrose supplemented with 300 mg l<sup>-1</sup> Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O, 200 mg l<sup>-1</sup> MgSO<sub>4</sub> 7H<sub>2</sub>O, 100 mg l<sup>-1</sup> KNO<sub>3</sub> and 100 mg l<sup>-1</sup> boric acid. Booklets soaked in 50 ml of medium kept in a large Petri dish for 15 minutes, were removed and excess medium allowed to drain. These were then placed in 80 mm Petri dishes and the top sheet of the filter paper carefully removed to expose the cellophane surface, blotted with a strip of dry filter paper. Pollen was carefully dusted over the cellophane. The Petri dishes were then covered with their complementary lids, wrapped tightly using a thin polypropylene film and incubated at 25 ± 2° C in the dark for a duration of 4–6 hours, after which the cellophane strips were carefully separated from the booklets and placed on a clean microslide, exposing the germinated pollen present on the cellophane to the stain. Staining was done using a drop of versatile stain (Alexander, 1980). A cover slip was placed over the stained cellophane strip after which the preparations were sealed with an epoxy resin, for microscopic examination.

Three replicates each of fresh and cryopreserved pollen were prepared. Pollen samples were thawed from the cannisters by the rapid thawing process, after retrieval from the cryogenic phase. Pouches were transferred to polypropylene centrifuge tubes and placed on a water bath at 37 ± 2° C for 5 minutes, before sampling pollen for viability test. In each preparation, more than 400 pollen grains were scored for their viability, using a Leitz Neo-Promar projection microscope. Pollen grains, whose tube lengths were longer than the grain diameter, were considered as having germinated. Analysis of Variance was performed on arcsine conversions of percentage germination data, using a factorial completely randomised design.

#### *Fertility assessment*

Fertility of cryopreserved pollen was tested by controlled field pollinations. Flowers of the desired female parents were emasculated and tied with thread at the bud stage in order to avoid open pollinations. Cryopreserved pollen after thawing was applied on the

Table 1. *In vitro* germination of pollen of different gladiolus cultivars after 48 hours storage in liquid nitrogen

Cultivars	Fresh	48 hour	Mean
1. Apsara	58.90 (50.11)	61.59 (51.68)	60.24 (50.89)
2. Meera	56.58 (48.76)	56.90 (48.94)	56.70 (48.85)
3. Poonam	56.94 (48.97)	57.69 (49.41)	57.31 (49.19)
4. Sapna	58.86 (50.08)	55.63 (48.21)	57.24 (49.15)
5. Shobha	56.34 (48.61)	60.00 (50.75)	58.17 (49.68)
Mean	57.52 (49.30)	58.36 (49.80)	

Values in parentheses indicate transformed values.  
SEM and LSD values computed using transformed means.

	SEM	LSD (p = 0.05)
Cultivar (C)	0.449	NS
Duration (D)	0.284	NS
Interaction (C × D)	1.875	NS

Table 2. *In vitro* pollen germination of different gladiolus cultivars after different durations of cryostorage

Cultivars	Fresh	Stored	Mean
1. Apsara	58.90 (50.11)	33.95 (35.61)	46.42 (42.86)
2. Jowagenaar	56.37* (48.64)	54.07 (47.32)	55.22 (47.98)
3. Meera	56.90 (48.94)	60.79 (52.21)	58.85 (50.58)
4. Nazrana	60.53 (51.06)	62.68 (52.36)	61.60 (51.71)
Mean	58.17 (49.69)	52.87 (46.87)	

Values in parentheses indicate transformed values.  
SEM and LSD values computed using transformed means.

\* Viability of pollen collected in 1993.

	SEM	LSD (p = 0.05)
Cultivar (C)	0.721	NS
Duration (D)	0.509	NS
Interaction (C × D)	3.057	NS

receptive stigma. Pollinated flowers were immediately covered with butter paper bags. Crosses with freshly collected pollen of all treatment cultivars were carried out, using the respective male and female parents. Six crosses were conducted each with fresh and cryopreserved pollen (Table 3).

Capsule formation and seed set were recorded in all the crosses after allowing for normal development and maturity, which was followed by harvest of the capsules from each cross and an estimate of the number of seeds per capsule.

## Results

### Feasibility studies

Pollen samples belonging to the 5 cultivars 'Apsara', 'Meera', 'Poonam', 'Sapna' and 'Shobha' responded well to the 48 hour cryogenic exposure. There was no significant difference in the germination percentages of fresh and preserved pollen (Table 1).

### Effect of cryogenic preservation on *in vitro* germination

Cryopreserved pollen samples of 'Apsara' (502 days), 'Meera' (476 days) and 'Nazrana' (503 days) germinated *in vitro*, which were comparable for 'Meera' and 'Nazrana' with fresh pollen. A significant reduction in viability ( $P < 0.05$ ) was observed for 'Apsara' pollen which could retain only 33.95% germination (*in vitro*) after storage (Table 2). Pollen of 'Jowagenaar' cryopreserved for 3668 days germinated *in vitro* at par with fresh pollen. It may be observed that the viability comparison made for this cultivar was with fresh pollen collected and tested on the day, the field pollinations were carried out with cryopreserved pollen. The method for fresh pollen germination was not yet optimised in 1983.

### Effect of cryogenic preservation on pollen fertility

Pollen, which had been cryopreserved for various intervals was fertile, inducing normal fruit (capsule) and seed set (Table 3). Among the pollen cryopreserved for durations more than one year, 'Meera' pollen induced the highest average number of seeds per capsule when 'Apsara' was used as the seed parent. This was followed by cryopreserved 'Nazrana' pollen on 'Shobha'. Cryopreserved 'Apsara' pollen failed to induce

Table 3. Field pollination with fresh and cryostored pollen

Parent		Average seed	Average seed
Seed	Pollen	set per capsule (fresh pollen)	set per capsule (cryopreserved pollen)
Apsara	Meera (F.P)	3.00	–
Apsara	Meera (1 year LN)	–	29.75
Sapna	Meera (F.P)	22.29	–
Sapna	Meera (1 year LN)	–	13.00
Shobha	Meera (F.P)	27.50	–
Shobha	Meera (1 year LN)	–	14.67
Meera	Apsara (F.P)	7.00	–
Meera	Apsara (1 year LN)	–	0.0
Shobha	Nazrana (F.P)	8.29	–
Shobha	Nazrana (1 year LN)	–	23.33
Dhiraj	Jowagenaar (F.P)	1.67	–
Dhiraj	Jowagenaar (10 year LN)	–	12.63

LN – Liquid Nitrogen.

F.P – Fresh Pollen.

any seed set on 'Meera'. The fertilization efficiency was reduced to less than 50% of the 'Meera' × 'Apsara' cross. The fertility status of cryopreserved 'Jowagenaar' pollen, tested on the *Fusarium* wilt resistant hybrid 'Dhiraj', produced an average number of 12 seeds per capsule, indicating the retention of pollen fertility after long-term cryopreservation (Table 3).

## Discussion

The present study has shown that pollen grains from the different gladiolus cultivars can be successfully recovered after cryogenic storage (48 hours to 10 years) without significant loss in viability. The ability of gladiolus pollen to effect fertilization does not appear to be adversely influenced by cryopreservation as is evident from the seed formation among crosses using cryopreserved pollen. Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following compatible pollinations (Shivanna et al., 1991). Koopowitz et al. (1980) produced, on an average 70 seeds per capsule with pollen of different gladiolus species, frozen at - 40° C for 2 years. In many crops, *in vitro* pollen germination does not always correlate well with per cent seed set or seed number per fruit. Cryopreserved 'Apsara' pollen, recorded low viability *in vitro*, and could not induce fruit and seed set on

'Meera'. Although optimal fresh pollen viability profiles were recorded for 'Apsara' (58.90), the cause for *in vitro* viability reduction after long-term cryogenic freezing might be due to inherent genotypic differences in response to cryopreservation (Towill, 1985) and *in vitro* viability assessment technique itself (Stanley & Linskens, 1974; Shivanna & Johri, 1985) which may elicit a poor response after storage, under artificial conditions. An increase in post-storage viability and number of seeds set through stored pollen have been observed in pine, onion and in pollen of some fruit crops (Towill, 1985; Ganeshan, 1986; Alexander & Ganeshan, 1993) similar to the results reported for 'Meera' and 'Nazrana' in the present study. Such a phenomenon is reported to occur due to certain after ripening processes (Towill, 1985) and enhanced physiological maturity (Alexander & Ganeshan, 1993). The ability of gladiolus breeders to recover seeds through the use of cryopreserved pollen provides scope for gene conservation at the haploid stage for prolonged periods of time. Since gladiolus is a crop with a high level of heterozygosity, variability can be conserved at different stages, including the pollen stage.

Published reports on cryogenic storage of pollen in other ornamental species show a reduction in seed recovery in *Narcissus* (Bowes, 1990) after 352 days of cryogenic storage. Marchant et al. (1993) cryopreserved rose pollen for 8 weeks without any reduction in fertility levels. Rajasekharan & Ganeshan (1994)

cryopreserved rose pollen for durations upto one year, maintaining the viability and fertility status. Hughes & Lee (1991) showed that desiccation of *Clianthus* pollen was necessary before storing at about - 180° C (liquid nitrogen vapour phase). Perhaps, pre-storage desiccation of 'Apsara' pollen could have improved the post-storage viability profiles *in vitro* after an optimal level of hydration. Optimum moisture levels necessary to maintain viability at low temperatures for diverse pollen species have not been determined (Connor & Towill, 1993).

Conservation of the haploid gene pool can be accomplished by consolidation and cryogenic storage of pollen from different species and cultivars. Such a protocol, made available to breeders, would help to improve their breeding efficiency, in terms of identifying potential pollen parents and preserving them for fulfilling commercial requirements. Pollen storage is being examined for base collection as a means to preserve the haploid level genetic diversity of clonal crops to supplement classic clonal preservation methods (Connor & Towill, 1993). The ultimate aim of gladiolus pollen storage has been to establish a 'Pollen Cryobank' through which pollen of desired species/cultivars could be consolidated and obtained for breeding without any seasonal or geographic constraints.

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